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ENVIRONMENTAL AND RECOMBINANT MICROORGANISMS FOR BIOPHARMACEUTICALS PRODUCTION

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Abstract: Current, very fast development of genetic engineering and protein engineering (main segments of modern biotechnology) is a good way for commercially production of proteins, which benefit biopharmaceutical, the enzyme and agricultural industries. These products augment the fields of medicine, diagnostics, food, nutrition, detergents, textiles, leather, paper, pulp, polymers and plastics. Proteins with biopharmaceutical application are mainly clinical reagents, vaccines and drugs. During the last decade, the pharmaceutical biotechnology represents the fastest growing segment in the biotechnology sector. Production of recombinant proteins for use as pharmaceuticals, is a multi-billion dollar industry. One third of the biopharmaceuticals has come from microorganisms, such as *Escherichia coli* and yeast. The selection of expression systems depends on the size and biochemical status of proteins. Large proteins and proteins that require glycosylation are usually expressed in a mammalian cells, fungi or the baculovirus system. Smaller proteins are produced by prokaryotic cells. There are some very useful advantages for prokaryotic recombinant expression systems: it is easy of culture, very rapid cell growth with possibility of IPTG expression induction and quite simple product purification. On the other hand, for very large proteins, for S-S rich proteins and proteins which require post-translational modifications, bacteria, usually *E. coli* strains are not robust system. Better are yeasts with very popular species *Saccharomyces cerevisiae* and *Pichia pastoris*. This article presents the current application of microbes as production platforms for recombinant proteins and its genetic engineering for the use as biopharmaceuticals..

key words: bacteria, yeast, recombinant protein production (RPP), biopharmaceuticals.

1. Introduction

During the last decade, the pharmaceutical biotechnology represents the fastest growing segment in the biotechnology sector. The first drug available from this area in 1982 was insulin. In a near future, due a huge development in recombinant DNA technology, the number of drugs originating from biotechnology is expected to reach 50% of new chemicals (Staub et al., 2011). Today there are more than 300 biopharmaceutical proteins and antibodies on the market (Nielsen, 2013).

Recombinant DNA and hybridoma technologies are used to engineer biological systems to produce 1) recombinant forms of natural proteins (including human growth hormones, cytokines, and insulin), 2) derivatives of natural proteins and living systems (including protein muteins, viral-like-particle vaccines, cancer cell vaccines, immunotoxins, and IgG fusion proteins), 3) viral vectors, plasmid vectors, and small interfering RNAs that carry genes or genetic information for vaccination or gene-therapy, and 4) *in vivo* diagnostic and therapeutic monoclonal antibodies (Zhu, 2012).

Leader et al. (2008) presented a complete classification of all current use therapeutic proteins, based on pharmacological action: 1. Therapeutic proteins with enzymatic or regulatory activity (e. g., insulin, growth hormone and erythropoietin); 2. Those with special targeting activity (e. g., etanercept and abciximab); 3. Protein vaccines (e. g., hepatitis B surface antigen); 4. Protein diagnostics (e. g., glucagon and growth hormone releasing hormone).

Biopharmaceuticals account for approximately 10% of the pharmaceutical market in 2007, 20% of newly approved drugs in recent years, and 40% of new entities in the pipeline. In 2006, it was estimated that approximately 2500 biotech drugs were in the discovery phase, 900 in preclinical and over 1600 in clinical trials (Walsh and Jefferis, 2006; Lowe and Jones, 2007; Karg and Kallio, 2009). Sales of many top-selling biopharmaceuticals are increasing, with US sales of the monoclonal antibody Herceptin used for the treatment of breast cancer growing by 82% from 2005 to 2006 alone (Lawrence, 2007). In 2007, the three top-selling pharmaceutical products sold by the Swiss drug-maker Roche were therapeutic monoclonal antibodies (Rituxan/Mabthera, Herceptin, and Avastin), contributing to sales of 9311 million € (Roche, Annual Finance Report for 2007). The total sales with monoclonal antibodies reached approximately 20 million € (2007) and the market

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volume is estimated to grow to about 38 million € annually by 2013 (Karg and Kallio, 2009).

This article presents the current application of microorganisms as production platforms for recombinant proteins and its genetic engineering for use as biopharmaceuticals.

2. Microbial systems for recombinant proteins production (RPP)

Low cost and simplicity of cultivating bacteria make the *Escherichia coli* expression system a preferable choice for production of therapeutic proteins both on a lab scale and in industry (Kamionka, 2011). One of the key advantages of *E. coli* is that the microorganism can be grown to high cell densities in appropriate bioreactors by high cell density cultivation (HCDC) techniques, which allow the production of high amounts of heterologous protein (Ni and Chen, 2009; Yoon et al., 2010; Waegeman and Soetaert, 2011).

To date, yeast strains have been extensively applied as hosts both for the production of biopharmaceuticals and industrial enzymes, too. As non-pathogenic organisms with a long history in agriculture and the food industry, yeasts have been the first choice hosts in many commercial applications (Gellissen et al., 1992).

Currently, most biopharmaceuticals produced are recombinant. The first step is a selection of expressions systems for protein amplification. There are some important factors for consideration during the choosing the right expression systems: protein quality, functionality, production speed and yield. *E. coli* strains or yeast constitute 40% of the therapeutic protein market and are usually choose for non-glycosylated proteins, because they are generally unable to provide mammalian glycosylation. Table 1 presented selected bio-pharmaceuticals which were produced with using of bacteria and yeast.

E. coli was the earliest platform to be exploited, and is still nowadays the most used production platform for recombinant proteins (Choi and Lee., 2004; Choi et al., 2006; Terpe 2006). Bacteria have been considered to be the most efficient producers of heterologous proteins due to several reasons: firstly, well developed molecular tools for genetic manipulation, secondly, annotated genomes and metabolic pathways, thirdly, high cell density cultivation capacity and growth rate and fourthly, high yield of recombinant proteins, up to 80% of its dry weight (Panda, 2003; Shumann and Ferreira, 2004; Tripathi et al., 2009; Porro et al., 2011). There are some limitations for production of human proteins with application of prokaryotic systems: bacteria are unable to perform some of the complex post-translational modifications – many proteins require further processing to become fully active (Ferrer-Miralles et al., 2009). However, the popular methylotrophic yeast – *Pichia pastoris*, on the way of genetic engineering are able to produce a human type of glycosylation. Proteins which demand N-glycosylation are usually made in mammalian cells which mimic human glycosylation (Demain and Vaishnav, 2009; Karg and Kallio, 2009; Swiech et al., 2012). In particular glycosylations that are needed to ensure proper function and activity, by influencing proper charge, solubility, folding, serum half live of the protein, *in vivo* activity, correct cellular targeting and immunogenicity, among others, cannot be often be fully accomplished in bacterial systems (Walsh and Jefferis 2006; De Pourcq et al., 2010; Martinez et al., 2012). The N-glycosylation pathway has been engineered in *E. coli*, and the progress towards the first

Table 1. Selected biopharmaceuticals which were produced with using of bacteria and yeast (adapted from Zhu, 2011; Huang et al., 2012; Swiech et al., 2012).

Product	Indication	Expression system	Year approved	Manufacturer
Pegloticase (Krytexxa)	Chronic refractory gout	Bacteria	2010	Savient
Victoza (Liraglutide)	Diabetes	Yeast	2010	Novo Nordisk
Xeomin (Incobotulinumtoxin A)				
Menveo (Meningitis vaccine)	Prevention of invasive meningococcal disease	Bacteria	2010	Novartis
Xiaflex (Collagenase)	Dupuytrenśs disease	Bacteria	2010	Auxilium
Prevnar 13	Pneumoniae	Bacteria	2010	Wyeth
Cimzia (Certolizumab Pegol)	Crohńs disease	Bacteria	2008	UCB
Nplate (Romiplostim)	Chronic immune thrombocytopenia purpura	Bacteria	2008	Amgen
PEG interferon alfa-2b (PegIntron)	Chronic hepatitis C infection Multiple sclerosis	*E. coli*	2008	Schering-Plough Novartis
Interferon beta 1b (Extavia)		*E. coli*	2009	

humanized glycoprotein produced from *E. coli* looks promising (Pandhal et al., 2010; Huang et al., 2012).

In addition straightforward recombinant DNA technology offers engineering tools to produce protein molecules with modified features. The lack of post-translational modification mechanisms in bacterial cells such as glycosylation, proteolytic protein maturation or limited capacity for formation of disulfide bridges may, to a certain extent, be overcome with protein engineering. Protein engineering is also often employed to improve protein stability or to modulate its biological action. More sophisticated modifications may be achieved by genetic fusions of two proteins (Kamionka, 2011).

3. Vector design and cytoplasmic expression of recombinant therapeutics

3.1. E. coli

E. coli K12 and its derivatives are the main strains used in recombinant therapeutic production in the biotech industry. A big advantage using *E. coli* K12 was given by the National Institutes of Health when it made this strain the standard and provided guidelines for safety. In addition, large-scale industrial production with *E. coli* requires approval by the local Biosafety Authority, which may be reluctant to approve other *E. coli* strains without the same safety level as *E. coli* K12. Common K12 derivatives used in the biotech industry include *E. coli* RV308 and W3110 (Furman et al., 1987; Chen et al., 2004; Huang et al., 2012).

Optimal gene transcription is normally a function of both gene dosage (plasmid) and promoter functionality. The productivity of recombinant protein is known to be affected by plasmid copy number and its structural and segregational stability. Choosing the optimal plasmid copy number is critical. Too low of a copy number will result in a low mRNA pool, as well as low protein productivity. A high copy number generally leads to high productivity; however, it also tends to impose metabolic burdens on cells. The plasmid copy number depends largely on the replication of origin, which dictates either flexible or rigid control over a plasmid. Both high copy number plasmids (e.g., pUC, 500–700 copies) and medium copy number plasmids (e.g., pBR322, 15–20 copies) have been used for therapeutic production in *E. coli* (Chen et al., 2004; Jana and Deb, 2005; Huang et al., 2012).

An ideal expression system is critical for high-level therapeutic production in *E. coli* to allow tightly regulated and efficient transcription. Choosing an appropriate vector system is largely dictated by the strength and the control of its promoter. To ensure high-level expression, a promoter with certain characteristics must be incorporated into the plasmid (Jana and Deb, 2005). For example, the promoter has to be strong to allow the recombinant protein production to account for 10–30% or more of total cellular protein. It should also be tightly regulated with limited basal expression in the non-induced state. Leaky expression can cause metabolic burdens on the cells during the growth period by diverting the carbon and energy source to premature protein formation. This situation can be detrimental when the expressed protein is highly toxic. In addition, some promoters must be used within specific *E. coli* strains to achieve optimal protein expression. Other important considerations are that the induction method should be simple and cost-effective, and, in most cases, the induction must be independent of the media components (Anné et al., 2012; Huang et al., 2012).

Successfully used promoters for different recombinant proteins production are *lac* and its synthetic derivatives, *tac* and *trc*, both in basic research and industry. *Tac* and *trc* promoters are stronger than *lac*, and all of them are induced by isopropyl-β-D-thiogalactopyranoside (IPTG). IPTG is used to derepress the lac repressor, but is expensive and toxic to some *E. coli* strains. To deal with a thermosensitive lac repressor mutant is available to induce protein expression by shifting temperature instead of using IPTG (Andrews et al., 1996; Jana and Deb, 2005; Graumann and Premstaller, 2006; Huang et al., 2012).

Recombinant DNA technology allows the expression of valuable heterologous proteins at high expression rates. Particularly in *E. coli* overexpression of proteins often leads to aggregation and deposition in dense, insoluble particles within the host cell, so-called inclusion bodies (IBs). They are easily distinguishable from other cell components due to their refractile character (Jungbauer et al., 2004; Jungbauer and Kaar, 2007; Jungbauer, 2012).

Many commercial and developmental therapeutics, such as interferons, interleukins and Fc-fusion proteins, are produced as IBs because of the multiple advantages of these protein aggregates (Graumann and Premstaller, 2006). High-yield production and versatility to express different proteins are two advantages associated with IBs formation (Luo et al., 2005). IBs are also stable protein aggregates and are resistant to protease activities *in vivo*. In addition, proteomic analysis showed that IBs are relatively homogeneous in composition, and, in some cases, the recombinant protein can account for more than 90% of the total imbedded polypeptides (Ventura and Villaverde, 2006). During the downstream processing, IBs can be easily isolated after cell disruption, and the resultant IB paste can be stored frozen for several months, providing manufacturing flexibility. Together, these characteristics allow IBs to be produced at high yield, as well as isolated and purified with simple and minimal efforts. However, this method also has its downside. Especially, the refolding of IBs to active protein represents a challenge, because efficient and high-yield refolding requires considerable optimization for each target protein. Resolubilization of IBs using chaotropic agents may also affect the integrity of the refolded proteins (Sahdev et al., 2008). Even so, acceptable recovery usually can be achieved at large industrial scale by using established strategies (Lilie et al., 1998; Eiberle and Jungbauer, 2010).

3.2. Bacillus strains

Recently, in academia and industry, more attention has been devoted to the use of Gram-positive *Bacillus spp*. strains for the production of recombinant proteins. *Bacillus spp*. strains share a number of advantages with *E. coli*, but become more and more attractive because of their ability to secrete the recombinant protein into the culture medium in high amounts. Furthermore, *Bacillus strains* are Gram-positive bacteria and hence do not contain an outer membrane which consists of lipopolysaccharides (LPS). These structures often contain endotoxins which are pyrogenic to humans. Disadvantages and points of particular interest are plasmid instability, the availability of expression vectors, protease activity, and the difficulty of high cell density cultivation (Porro et al., 2011; Waegeman and Soetaert, 2011). The most often used strains include *Bacillus megaterium*, *Bacillus subtilis*, and *Bacillus brevis* (Terpe, 2006).

Bacillus subtilis is a rod-shaped, soil bacterium that secretes numerous enzymes to degrade a variety of substrates, enabling the bacterium to survive in a continuously changing environment. These enzymes are produced commercially and this production represents about 60% of the industrial-enzyme market. Unfortunately, the secretion of heterologous proteins, originating from Gram-negative bacteria or from eukaryotes, is often severely hampered. Several bottlenecks in the *B. subtilis* secretion pathway, such as poor targeting to the translocase, degradation of the secretory protein, and incorrect folding, have been revealed. Nevertheless, research into the mechanisms and control of the secretion pathways will lead to improved *Bacillus* protein secretion systems and broaden the applications as industrial production host (Westers et al., 2004).

3.3. 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 *Pfenex* 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).

Other hosts that have been exploited for RPP include *Pseudomonas putida* for antibody fragments (Dammeyer et al., 2011). Many more bacterial systems are described, but more than often the limited information about their genetics or metabolism or the unavailability of expression vectors or promoter systems hinders the expansion of their application (Waegeman and Soetaert, 2011).

3.4. Yeast expression systems

Yeast expression systems are an attractive choice, also provide higher protein titers (>1 g/L) in only few days fermentation processes. Additionally, recent advances in genetic and metabolic engineering, and tools in genomics and systems biology could make *Saccharomyces cerevisiae* a preferred production platform for a range of pharmaceutical proteins (Gerngross, 2004; Hou et al., 2012). Very promising attempts have been recently achieved to introduce human glycosylation patterns in yeast (humanized yeast platforms). The main biopharmaceuticals produces by *S. cerevisiae* are insulin (and insulin analogs), human serum albumin, hepatitis vaccines and virus like particles, e. g., for vaccination against human papillomavirus (Nielsen, 2013). The state-of-the-art methodologies and approaches show that *S. cerevisiae* can be engineered to become an even better producer for a wider range of pharmaceutical and blood proteins. Compared to *E. coli*, heterologous proteins produced in *S. cerevisiae* do not have methionine modification which affects the biological function of the rHb. Compared to plant and animal expression systems, the yeast system is cheaper and faster to manipulate. In the work of Martinez et al., 2012 several potential strategies for increasing human hemoglobin production in S. *cerevisiae*, for example, globin folding, heme uptake, and subunit assembling were proposed. The recent advances in the field of metabolic engineering allow *S. cerevisiae* to become an efficient cell factory for the production of heterologous proteins (Martinez et al., 2012).

Pichia species have been successfully engineered to produce specific human-like glycoforms of proteins; however, recent advances reported in this field in *S. cerevisiae* seem to indicate that, with further development, it may increase the array of strains available that are able to produce human-type glycosylated proteins, and these strains will become a valuable platform for the production of glycoproteins for therapeutic use (De Pourcq et al., 2010; Amano et al., 2008; Chigira et al., 2008; Martinez et al., 2012).

The major advantages of *E. coli* and yeast expression systems are presented in Table 2.

Examples of bacterial and yeast expression systems are presented in Fig. 1 and Fig. 2.

4. Conclusion

After 30 years after the first recombinant insulin was approved by the FDA, *E. coli* and yeast are still widely used by the biotech industry for biopharmaceuticals production. Today there are more than 300 biopharmaceutical proteins and antibodies on the market and *E. coli* strains or yeast constitute 40% of the therapeutic protein market. These microorganisms are usually choose for non-glycosylated proteins, because they are generally unable to provide mammalian glycosylation. Recent advances in genetic and metabolic

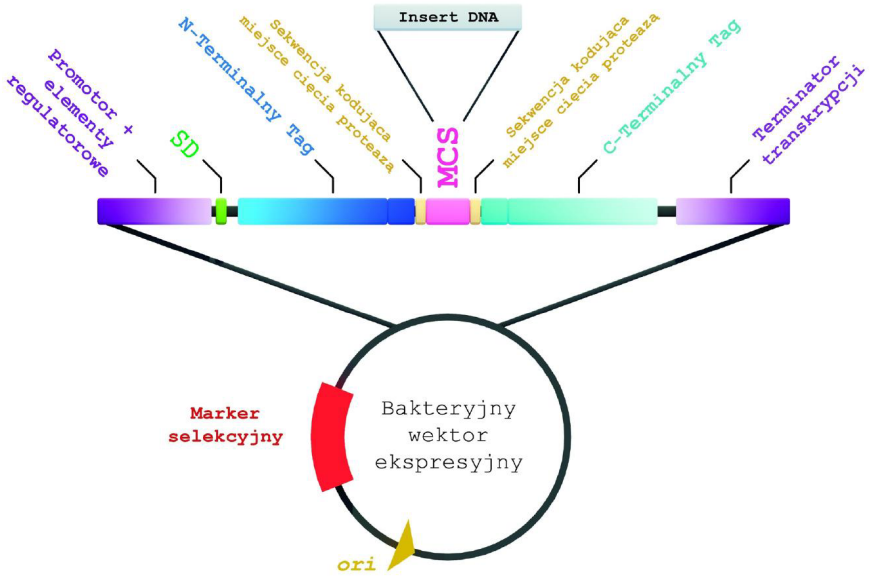

Fig. 1. Bacterial expression systems.

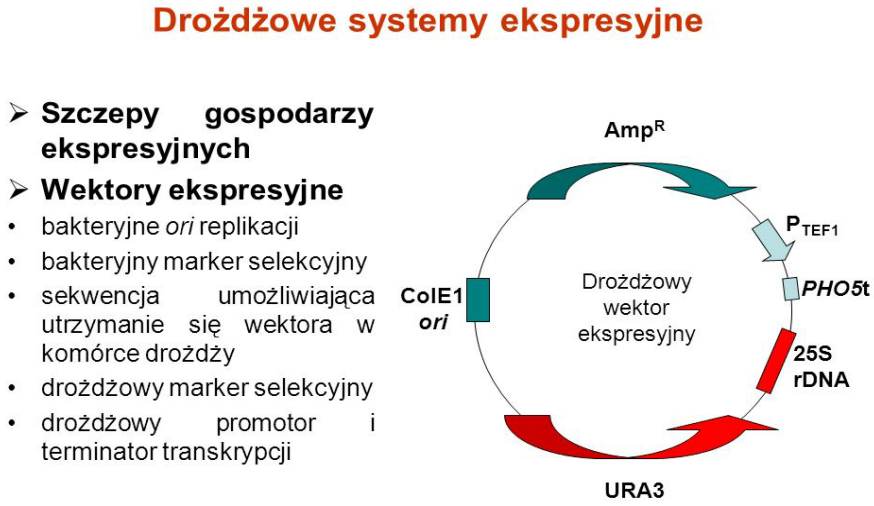

Fig. 2. Yeast expression systems.

Table 2. The major advantages of *E. coli* and yeast expression systems (according to Demain and Vaishnav, 2009).

E. coli expression systems	Yeast expression systems
Rapid growth	Rapid growth and high yield
Rapid expression	Stable production strains
Promoter control is not difficult	Durability
High product yields	Cost effective
Good understood genetics	High density growth
Ease of culture and genome modification	High productivity
Inexpensive	Suitability for production of isotopically-labeled protein
Mass production fast and cost effective	Product processing similar to mammalian cells Can handle S-S rich proteins Can assist protein folding Can glycosylate proteins

engineering, and tools in genomics and systems biology could introduce human glycosylation patterns in yeast (humanized yeast platforms) and create preferred production platform for a range of pharmaceutical proteins. The N-glycosylation pathway has been engineered in *E. coli*, and the progress towards the first humanized glycoprotein produced from *E. coli* looks promising.

Biotechnological progress in the field of metabolic engineering allows bacteria and yeast to become an efficient cell factory for the production of heterologous proteins, which are very important and often are the only way for life saving medical human therapy.

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