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# Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation

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17 **Abstract**

18

19 Since the approval of recombinant insulin from *Escherichia coli* for its clinical use in the early 80's, the
20 amount of recombinant pharmaceutical proteins obtained by microbial fermentations has
21 significantly increased. The recent advances in genomics together with high through-put analysis
22 techniques (the so-called –omics approaches) and integrative approaches (systems biology) allow the
23 development of novel microbial cell factories as valuable platforms for large scale production of
24 therapeutic proteins. This review summarizes the main achievements and the current situation in the
25 field of recombinant therapeutics using yeast *Saccharomyces cerevisiae* as a model platform, and
26 discusses the future potential of this platform for production of blood proteins and substitutes.

27 **Introduction**

28

29 Microorganisms have been extensively used since ancient times for the production of fermented
30 food and beverages, thousands of years before the actual nature of the fermentative processes was
31 known. In the early 20$^{th}$ century the production of citric acid based on microbial fermentation was
32 initiated as the first large scale fermentation product and this was followed by industrial production
33 of penicillin as the first antibiotic. Introduction of the genetic engineering in the 70's paved the way
34 for the establishment and development of the current biotech industry, allowing the commercial
35 production of industrial enzymes and biopharmaceutical proteins. In 1980, the FDA approved for
36 clinical use the recombinant insulin obtained from *E. coli*, becoming the first recombinant
37 pharmaceutical protein to enter the market [1]. Since then, the biotechnology industry has grown
38 substantially, and currently about 25% of commercial pharmaceuticals are biopharmaceuticals [2]
39 with 2010 sales exceeding USD100 billions [3]. About half of the world-wide sales are in the USA with
40 monoclonal antibodies representing the majority (>USD18 billions) followed by hormones (USD11
41 billions) and growth factors (>USD10 billions)[4]. Together with the production of industrial enzymes,
42 the recombinant protein production market is expected to rise to 169 billion dollars in 2014 [3]
43 (Figure 1).

44

45 **Platforms for production of pharmaceutical proteins**

46

47 Industrial biotechnology has traditionally used numerous bacterial and eukaryal cells as production
48 platforms, with the main criterion for host selection being the ability to produce the desired
49 compound. However, with the advent of genetic engineering it became possible to introduce
50 heterologous genes and create new traits in non-natural producers, allowing the development of cell
51 factories for the production of chemicals through metabolic engineering. *E. coli* was the earliest

52 platform to be exploited, and is still nowadays the most used production platform for recombinant
53 proteins [5], covering approx. 30% of the total production of recombinant proteins [1] (Figure 1). In
54 general terms, bacteria have been considered to be the most efficient producers of heterologous
55 proteins due to several reasons: i) well developed molecular tools for genetic manipulation, ii)
56 annotated genomes and metabolic pathways, iii) high cell density cultivation capacity and growth
57 rate and iv) high yield of recombinant proteins, up to 80% of its dry weight [6-8]. However, standard
58 prokaryotic systems have some limitations for production of human proteins. For example, bacteria
59 are unable to perform some of the complex post-translational modifications [1], which itself
60 represents a limitation, since many proteins require further processing to become fully active. In
61 particular glycosylations that are needed to ensure proper function and activity, by influencing
62 proper charge, solubility, folding, serum half live of the protein, *in vivo* activity, correct cellular
63 targeting and immunogenicity, among others, cannot be often be fully accomplished in bacterial
64 systems [9] [10]. These limitations have paved the way towards eukaryotic expression systems and
65 there exists several eukaryotic systems that are currently in use for large scale production of
66 different therapeutic proteins (Table 1), with the most studied being hybridoma cells, Chinese
67 Hamster Ovary (CHO) cells [11], insect cells [12,13] and yeast cells [14]. Mammalian systems like
68 hybridoma and CHO cells clearly have the highest similarity to human cells, and proteins produced by
69 these systems are often properly folded and glycosylated. However the costs for their cultivation are
70 high (e.g. expensive media and growth factors, contaminations with microorganisms and viruses),
71 they have a limited secretion capacity and protein yields are usually low [15]. On the other hand
72 fungal expression systems, and in particular yeast, can grow in relatively cheap and defined media,
73 decreasing the production costs. Besides, they are not so susceptible to contaminations and in
74 addition, the yeast cells are less sensitive since the wall makes them more resistant to shear stress
75 during the production process [10]. Yeast expression systems also provide higher protein titers
76 (>1g/l) in fermentation processes that even last shorter time (only few days) [16]. Based on this, we
77 propose yeast as an attractive choice, and recent advances in genetic and metabolic engineering, and
78 tools in genomics and systems biology could make *S. cerevisiae* a preferred production platform for a
79 range of pharmaceutical proteins[17]. However, even though yeasts are eukaryotic systems, the
80 glycosylations of proteins may differ substantially from that performed by mammalian cells, a
81 difference that can be in some cases detrimental for its subsequent therapeutic use. N-glycosylation
82 in yeast, for example, is of the high mannose type whereas human N-glycans are mainly of the
83 complex or hybrid type. In addition to N-glycosylation, yeast O-glycosylation characterized by shorter
84 glycan structures, also differs from the human type, which is mucin-type in contrast to the
85 oligomannosyl-glycans in yeasts [10]. Very promising attempts have been recently achieved to
86 introduce human glycosylation patterns in yeast (humanized yeast platforms). To date, only *Pichia*

87 species have been successfully engineered to produce specific human-like glycoforms of proteins,
88 however recent advances reported in this field in *S. cerevisiae* seem to indicate that, with further
89 development, it may increase the array of strains available that are able to produce human-type
90 glycosylated proteins, and these strains will become a valuable platform for the production of
91 glycoproteins for therapeutic use [10,18,19].

92

93 **How to make *Saccharomyces cerevisiae* a better producer of pharmaceutical proteins?**

94

95

96 The technology for industrial production of recombinant pharmaceutical proteins in *S. cerevisiae* is
97 well established and currently applied for production of human insulin, hepatitis virus vaccines and
98 human papilloma virus vaccines, and its potential to be used for large scale production of many other
99 proteins in the forthcoming years is therefore high. Furthermore, the advent of systems biology
100 allowing global metabolism analysis and the application of so-called "omics" approaches such as
101 transcriptome, proteome and metabolome data, facilitates the identification of the bottlenecks and
102 factors limiting the full potential of this yeast to become a better producer [20,21], and consequently
103 the application of metabolic engineering to overcome constraints in productivity could definitely
104 allow the establishment of *S. cerevisiae* as a suitable platform for large scale production of
105 heterologous (including human) proteins [22].

106

107 There are several reports describing how either genetic or metabolic engineering can be successfully
108 performed in *S. cerevisiae* [22,23] resulting in the generation of strains showing an enhanced
109 production capacity of heterologous proteins [17,24,25]. Often just introducing an entire new
110 pathway for the production of the desired compound does not result in high levels of production.
111 because protein folding and secretion can represent the major limitation in terms of protein yields in
112 yeast [20]. Folding and secretion are complex processes and the molecular machineries are
113 composed of large number of components, so further modifications and development of these
114 pathways requires integrative analysis of the whole secretory pathway. Such approach has been
115 successfully carried out by engineering different elements of the secretory pathway, and by
116 combining different expression systems in order to optimize the production of several kinds of
117 different proteins showing different biochemical properties (i.e. size, type of modification
118 (glycosylation and/or disulfide bond formation)), such as human insulin precursor or α-amylase
119 [17,26,27]. Through a combination of these approaches it is possible to select the best protein
120 producers for further optimization, and this may lead to generic protein producing strains that can be
121 used as general platforms for the production of bio-based pharmaceutical proteins (Figure 2).

122 **Production of Recombinant Human Blood Proteins**

123 Among the 58 biopharmaceuticals approved in the United States and/or Europe from 2006 to 2010
124 four are blood related proteins, including a rh coagulation factor VIII produced in CHO cells, a rh
125 antithrombin from milk of transgenic goats, a plasma kallikrein inhibitor produced in *Pichia. pastoris*,
126 and a rh thrombin produced in CHO cells [28]. All have therapeutic use for treatment of hemophilia.
127 To date, most of the recombinant blood related biopharmaceuticals approved for clinical treatment
128 are coagulation factors, including factor VIII, factor VIIa, and factor XI [28] with recombinant human
129 serum albumin (rHSA) as an exception. Due to the fact that it is not glycosylated, a variety of
130 expression hosts have been screened to express rHSA, including bacterium *Bacillus subtilis*, yeast *S.*
131 *cerevisiae, Kluyveromyces. lactis* and *P. pastoris* etc [29]. So far, Novozymes has commercialized two
132 rHSA produced by *S. cerevisiae*, namely Recombumin and Albucult. Since the clinical dosage of HSA is
133 usually quite high, normally over 10g/L, many studies have tried to express rHSA also in *P. pastoris*
134 for its high capacity in heterologous protein production [30]. The rHSA produced from *P. pastoris* has
135 gone through the clinical trials and confirmed the safety and efficacy to treat different diseases [31].
136
137 Aside from rHSA, many other human blood proteins are also under active studies [32-34]. Human
138 fibrinogen (Hf) is a large plasma glycoprotein that plays a critical role in the last stage of coagulation.
139 It is dimeric and comprised of two sets of three different polypeptides, namely Aα, Bβ and γ. The
140 protein was expressed in *P. pastoris* protease deficient strain by constructing an expression vector
141 containing the cDNA of three individual peptide chains. Even though the peptides expressed were of
142 different N-glycosylation patterns as that of native Hf, they were correctly assembled to a functional
143 rHf that is capable of forming a clot in the presence of factor XIIIa [32]. Human α-1-antitrypsin
144 (hAAT) was produced in tomato: the codon modified cDNA sequence was expressed and the mRNA
145 5' and 3' flanking regions were modified to achieve a high-level expression by eliminating mRNA
146 destabilizing sequences, which are ATTTA and its variants, splice sites and A/T strings. In contrast to
147 the unglycosylated rAAT expressed in *E. coli*, the glycosylated rATT from transgenic tomato was
148 biologically active [33]. One more example is the production of human transferrins (Tf) which are a
149 family of monomeric proteins that are of different sizes depending on the extent of glycosylation.
150 Besides its central role to facilitate iron transport and metabolism, a lot more other functions have
151 been evidenced, e.g. acting as a growth factor for mammalian tissue cells, as a neurotropic factor
152 during neural stem cell development and as an angiogenic factor to promote endothelial cell
153 migration etc, enabling a development of many novel practical applications in medicine [34]. To date,
154 several heterologous systems including *E. coli*, yeast, transgenic plants, mammalian and insect cells
155 have been developed for rhTf production [34], among which *E. coli* was reported to be inefficient due
156 to the production of inactive hTf. Successful expressions of hTf were reported in *S.cerevisiae* and *P.*

157 *pastoris* with the resulting proteins being functional independent of use of only a partial sequence or
158 a full sequence, or with mutated glycosylation sites [34].
159

160 ***S. cerevisiae* as a cell factory for human hemoglobin production**

161 All the proteins described above are blood plasma components which contribute to different roles of
162 blood such as coagulation, clotting, transport of iron, maintain blood osmotic pressure and blood
163 volume. The additional crucial role of blood is the transport of oxygen and the only component in
164 blood that possesses oxygen carrier function is hemoglobin (found in erythorcytes), and this is
165 therefore a key component for development of human blood substitutes for treatment of patients
166 with injuries, anemia or in post-operational recovery.

167 Production of recombinant hemoglobin (rHb) has been attempted since the late 80's. A variety of
168 strategies have been applied using several different expression systems, ranging from bacteria to
169 higher organisms such as transgenic plants and animals [35]. The work was first done in *E. coli*, where
170 a single β globin was expressed with a cleavable linker and refolded *in vitro* with native α globin and
171 exogenous heme [35]. The work was quite laborious and researchers therefore tried to express α and
172 β globins simultaneously *in vivo* with endogenous heme incorporated [36]. It was observed that the
173 essential parameters for normal human hemoglobin, namely Bohr effect and 2,3-BPG effects of the
174 rHb were reduced which very likely resulted from the methionine termini at the end of the globin
175 chains [37]. The amount of methionine modified α and β globins were significantly reduced by co-
176 expression of the methionine amino-peptidase (Met-AP) gene with the globin genes resulted in an
177 increased yield of rHb [36]. Further optimizations of the *E. coli* expression system included codon
178 optimization for globin expression in a T7 promoter system [38]; Site-directed mutations in β globin
179 chain in order to reduce the extreme oxygen affinity to rHb (no release of oxygen) due to the lack of
180 2, 3-BPG allosteric regulation; and a tandem fused α globin to prevent the dissociating of the
181 tetramer into a αβ dimer [39]. In some resent studies, researchers have tried to co-express α globin
182 with its molecular chaperon, α-hemoglobin stabilizing protein (AHSP) and revealed its mechanisms
183 on preventing α globin precipitation. [40-42]. Correct expression and folding of human Hb have also
184 been accomplished in animals, e.g. pig [43] and mice [44,45], and in plants, e.g. tobacco[46].
185

186 The previous examples and the state-of-the-art methodologies and approaches show that *S.*
187 *cerevisiae* can be engineered to become an even better producer for a wider range of
188 pharmaceutical and blood proteins. Comparing to *E. coli*, heterologous proteins produced in *S.*
189 *cerevisiae* do not have methionine modification which affecting the biological function of the rHb.

Compared to plant and animal expression systems, the yeast system is cheaper and faster to manipulate. As an outlook, we propose several potential strategies for increasing human hemoglobin production in *S. cerevisiae* e.g. globin folding, heme uptake, and subunit assembling.

Additionally to the existing strategies such as site directed mutagenesis of the amino acids that are important for stabilization [47,48], co-overexpression of α and β globin genes, cross-linking two α globins as di-α to avoid αβ dimer formation, it is worth trying to co-express the AHSP gene together with the α and β globin genes to increase the α globin stability [42,49,50] thus further enhancing the production of rHb. It has also been reported that heme can accelerate hemoglobin accumulation in immature cultured erythroid cells [51] and heme is not only the indispensable prosthetic group but also essentially involved in assembling and ensuring a stable tetramer structure [52]. As heme supplementation is expensive and the mechanism behind is poorly understood [53], engineering the heme synthesis pathway is proposed to be a better choice to increase heme levels in the cell. Examples for engineering the heme biosynthesic pathway include over-expression of the rate-limiting enzymes in the synthesis pathway [54], engineering ALA (aminolevulinic acid) synthesis since it is the first intermediate involved in heme synthesis, as well as engineering the iron uptake pathway.

In conclusion, the recent advances in the field of metabolic engineering allowed that *S. cerevisiae* become an efficient cell factory for the production of heterologous proteins. By a systems biology approach, further improvements might be implemented through integrative analysis and the development of mathematical predictive models, being this yeast expected to become the suitable platform for sustainable large scale production of protein therapeutics in the forthcoming future.

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225 *of special interest

226 **of outstanding interest

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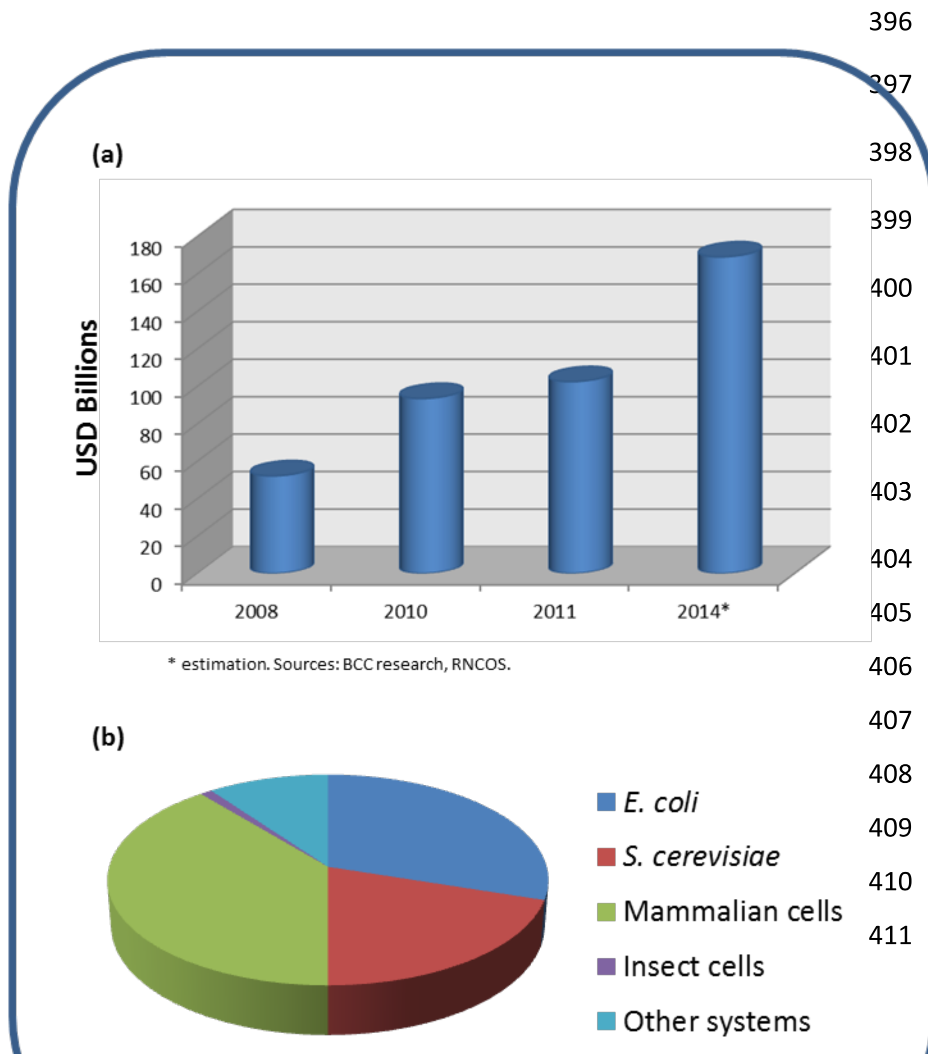

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423 Figure 1. (A) Global market for recombinant protein drugs and (B) percentage of protein-based
424 recombinant pharmaceuticals, produced by different systems [1].
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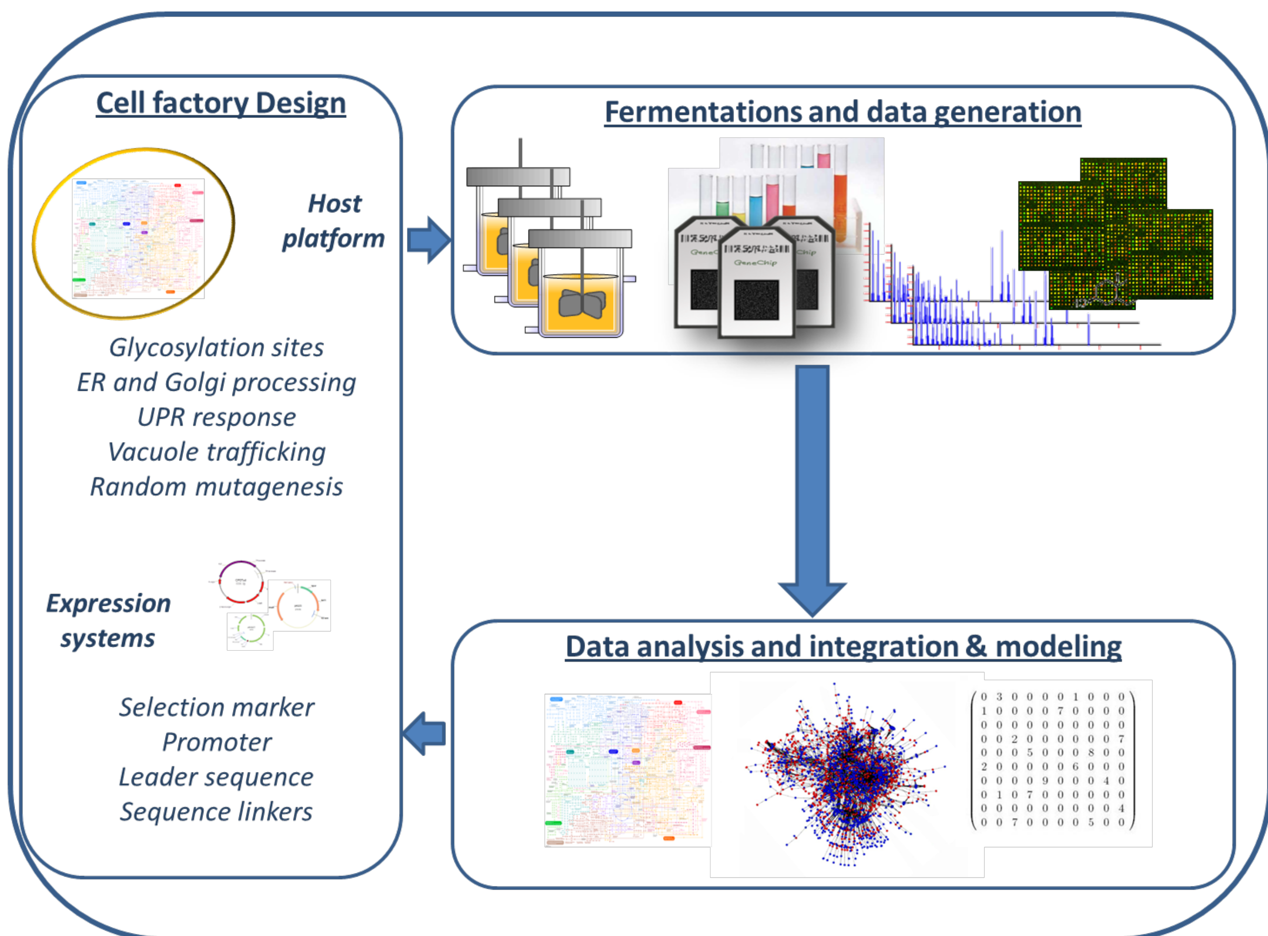


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428 Figure 2. Workflow overview for the development of yeast cell factories by metabolic engineering
429 from a systems biology approach. Engineering for protein production improvement can be
430 implemented either at a host level (e.g. metabolic engineering of gene pathways related to different
431 steps concerning protein processing and secretion) and/or be applied to the improvement of
432 expression systems by addition/testing of different features (e.g. the suitable selection marker for
433 each system, promoter sequences, etc). High throughput analysis methodologies allow then the
434 generation or large data sets, which can be processed and integrated in mathematical models for the
435 identification of new potential targets, allowing further improvement via retrofitting the system, and
436 therefore resulting in an enhanced protein production capability of the cell platform.
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| **Protein** | **System** | **Production level** | **Refs** |
|---|---|---|---|
| Hirudin | *S. cerevisiae* (Y) | 60 mg/L | [55] |
| | *H. polymorpha* (Y) | - | [56] |
| Interferon α-2b | *H. polymorpha* (Y) | 120 mg/L | [57] |
| Hepatitis B vaccine | *H. polymorpha* (Y) | - | [58] |
| Angiostatin | *P. pastoris* (Y) | 108 mg/L | [59] |
| Anti-HBs Fab | *P. pastoris* (Y) | 50 mg/L | [60] |
| Human serum albumin | *K. lactis* (Y) | 3 g/L | [61] |
| | *S. cerevisiae* (Y) | 3 g/L | [62] |
| | | 10 g/L | [63] |
| | *P. pastoris* (Y) | | |
| Human interleukin 6 | *A. niger* (F) | 150 mg/L | [64] |
| Human apolipoprotein AI | CHO cells (M) | 80 mg/ml | [65] |
| Insulin precursor | *P. pastoris* (Y) | 3 g/L | [66] |
| | *S. cerevisiae* (Y) | 98mg/L | [27] |
| Human tPA | CHO cells (M) | 34 mg/L | [67] |
| Human gonadotropin | CHO cells (M) | 3 g/L | [67] |
| Erythropoietin (epoetin α) | CHO cells (M) | - | [68] |

| | | | |
|---|---|---|---|
| Monoclonal Ab | NSO cells (M) | 3 g/L | [67] |
| HPV vaccine (Cervarix™) | Insect cells | - | [69] |
| Human proapolipoprotein AI | Insect cells | 80 mg/L | [70] |
| Clotting factor VII a | BHK cells (M) | - | [68] |

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445 Table 1. Examples of recombinant therapeutic proteins successfully expressed using different
446 production systems, including highest production levels reported in each organism. CHO = Chinese
447 hamster ovary cells; BHK = Baby hamster kidney cells; NSO = Myeloma cells; (Y) = Yeast; (F) =
448 Filamentous fungi; (M) = Mammalian.

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450 Hightlights

451 Recombinant therapeutic production is a multibillion dollar market.

452 *E. coli* represents 30% of recombinant protein production but not suitable for human therapeutics.

453 Eukaryotic systems other than yeast are costly or not so efficient regarding protein yields.

454 *S. cerevisiae* shows a high potential to be a suitable platform for therapeutic protein.

455 Human blood proteins are the next candidates to be challenged by *S. cerevisiae* system.

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