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Research review paper

Production of recombinant proteins by yeast cells

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ABSTRACT

Yeasts are widely used in production of recombinant proteins of medical or industrial interest. For each individual product, the most suitable expression system has to be identified and optimized, both on the genetic and fermentative level, by taking into account the properties of the product, the organism and the expression cassette. There is a wide range of important yeast expression hosts including the species *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica* and *Arxula adeninivorans*, with various characteristics such as being thermo-tolerant or halo-tolerant, rapidly reaching high cell densities or utilizing unusual carbon sources. Several strains were also engineered to have further advantages, such as humanized glycosylation pathways or lack of proteases. Additionally, with a large variety of vectors, promoters and selection markers to choose from, combined with the accumulated knowledge on industrial-scale fermentation techniques and the current advances in the post-genomic technology, it is possible to design more cost-effective expression systems in order to meet the increasing demand for recombinant proteins and glycoproteins. In this review, the present status of the main and most promising yeast expression systems is discussed.

Contents

1. Introduction

Yeast species have been popular industrial hosts for recombinant protein (r-protein) production because they combine the advantages of unicellular organisms (i.e., ease of genetic manipulation and rapid

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growth) with the ability to perform eukaryotic post-translational modifications. Unlike more complex eukaryotic organisms, yeast expression systems are economical, can rapidly reach high cell densities, produce high protein titers and do not contain pyrogens, pathogens or viral inclusions.

Designing the optimal system for r-protein production involves many crucial steps: (1) selecting the host strain that enables proper folding and post-translational modifications, (2) choosing a suitable vector (episomal or integrative) with an appropriate promoter (constitutive, inducible or repressible) and selectable marker, (3) codon-optimizing the gene (4) fusing the gene to an epitope tag if necessary for affinity purification or detection of the r-protein, (5) choosing the signal sequence to target the r-protein to the intracellular or extracellular medium, (6) preventing the proteolytic cleavage of the product, (7) designing the fermentation medium (carbon and nitrogen sources, induction conditions), and (8) optimizing the bioprocess parameters (temperature, pH, oxygen transfer).

Saccharomyces cerevisiae, the first and best characterized yeast expression system, was developed in the 1980s and highly benefited from its traditional use in baking, brewing and wine making. However, numerous cases of plasmid instability, low protein yields and the hyperglycosylation of proteins have limited the number of commercial products on the market from *S. cerevisiae* (Buckholz and Gleeson, 1991; Gellissen et al., 2005). Moreover, *S. cerevisiae* produces proteins with *N*-linked glycosylation terminated via α-1,3-linked mannose residues, which are considered to be allergenic.

These issues have led to the development of alternative expression systems that are now well established, including two methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha*, the budding yeast *Kluyveromyces lactis*, the fission yeast *Schizosaccharomyces pombe*, and two dimorphic yeast *Arxula adeninivorans* and *Yarrowia lipolytica*. The genome sequencing projects for *S. cerevisiae* (Goffeau et al., 1996), *S. pombe* (Wood et al., 2002), *H. polymorpha* (Ramezani-Rad et al., 2003), *K. lactis*, *Y. lipolytica* (Dujon et al., 2004), and *P. pastoris* (De Schutter et al., 2009; Mattanovich et al., 2009a) have resulted in a vast amount of data and various databases are available on the Web, such as *Saccharomyces* Genome Database (SGD) (Cherry et al., 1998), Yeast Proteome Database (YPD) (Hodges et al., 1999) and *Pichia pastoris* Genome Browser (Mattanovich et al., 2009b). In addition, host strains are being engineered to perform more humanized *N*-glycosylation, which was accomplished first in *P. pastoris* (Hamilton et al., 2006) followed by initial studies in *H. polymorpha* (Kim et al., 2006; Oh et al., 2008), *Y. lipolytica* (Song et al., 2007), *K. lactis* (Liu et al., 2009) and *S. pombe* (Ohashi et al., 2009), opening the route for yeast to become the major industrial hosts for therapeutic proteins.

Clearly, no single yeast expression system can provide all the desired properties for r-protein production. In order to benefit from the current availability of a wide range of host–vector combinations and to design the optimal system, this review focuses on the state-of-the-art developments and technological achievements in the yeast expression systems, providing a comparative analysis. Furthermore, future perspectives on r-protein production in yeast systems are discussed.

2. Host species: general characteristics

The choice of the host strain is of paramount importance for the success of the whole process. It has become evident that the compatibility of the host's biochemical environment with the ability to process and translate the RNA transcript, along with its ability to modify and sustain the translated protein are equally important in choosing the most appropriate host cell for protein expression (Greene, 2004). Moreover, through metabolic engineering (Ostergaard et al., 2000), evolutionary engineering (Sonderegger and Sauer, 2003) and using industrial systems biology toolbox (Papini et al., 2010), superior host strains are continuously being developed. General characteristics of the most popular yeast strains are described below.

2.1. Saccharomyces cerevisiae

S. cerevisiae, the traditional biotechnological organism and the first eukaryote whose genome was fully sequenced (Goffeau et al., 1996), has been engineered to express a wide variety of recombinant proteins for the last three decades. The first vaccine effective against human viral infections, hepatitis B, (McAleer et al., 1984) was produced intracellularly in recombinant *S. cerevisiae*. Still today, the recombinant therapeutics approved by the Food and Drug Agency (FDA) and the European Medicines Agency (EMEA) from microbial eukaryotic cells are almost exclusively produced by *S. cerevisiae* (Huang et al., 2010). Products on the market which are made in *S. cerevisiae* include insulin, hepatitis B surface antigen, urate oxidase, glucagons, granulocyte macrophage colony stimulating factor (GM-CSF), hirudin, and platelet-derived growth factor (Demain and Vaishnav, 2009).

In addition to the previously mentioned advantages of yeast systems, which are generally attributed to *S. cerevisiae*, the ongoing industrial popularity of this organism is mostly due to the fact that it has been recognized as generally regarded as safe (GRAS) strain, and more importantly to the accumulated knowledge on its physiology, genetics and fermentation techniques. The high-throughput data collected by functional genomic tools such as transcriptome analysis (Lashkari et al., 1997), proteome analysis (Usaite et al., 2008; Zhu et al., 2001), metabolome analysis (Jewett et al., 2006; Villas-Boas et al., 2005), flux analysis (Sauer, 2006), interactome analysis (Harbison et al., 2004; Lee, 2002; Uetz et al., 2000) and locasome analysis (Huh et al., 2003) are contributing to the set of valuable information, and *S. cerevisiae* is possibly the organism with the most comprehensive experimental dataset available (Petranovic et al., 2010). Competitiveness of this yeast is also due its high capacity to produce and consume ethanol (Crabtree positive), and a tolerance for environmental stress, such as low oxygen levels.

Among other yeast species, *S. cerevisiae* possesses the highest glycosylation capacity, leading to hyperglycosylation and a reduced secretion rate. Alternative recombinant protein expression systems are emerging with various advantages, while *S. cerevisiae* is still the predominant host used for metabolite productions and the model organism to study human proteins linked to genetic and degenerative diseases (Petranovic and Nielsen, 2008). Moreover, it has been recently shown to be useful as a probiotic in humans for oral delivery of therapeutic proteins (Blanquet et al., 2001; Omara et al., 2010).

2.2. Pichia pastoris

The methylotrophic yeast *P. pastoris* can use methanol as its sole carbon and energy source in the absence of a repressing carbon source. Derived from the alcohol oxidase 1 (AOX1) gene of methanol utilization pathway, *AOX1* promoter is known to be one of the strongest and tightly regulated eukaryotic promoters. A wide variety of proteins have been expressed using this promoter, with yields up to 14.8 g L^{-1} (of mouse collagen, Werten et al., 1999), and claims up to 20–30 g L^{-1} (Morrow, 2007) of recombinant protein. Since *P. pastoris* prefers a respiratory rather than a fermentative mode of growth, fermentation products such as ethanol and acetic acid do not build up quickly (Cereghino et al., 2002), enabling cultures to reach high cell densities (200 g L^{-1} dry weight, Heyland et al., 2010). There is a tendency of *P. pastoris* for secretion of proteins, even with high molecular weight; while in *S. cerevisiae* proteins are mostly retained in the periplasm. Moreover, simple purification of secreted proteins is possible due to the relatively low levels of endogenous proteins in the extracellular medium.

P. pastoris exist in the vegetative haploid state unless there is nitrogen limitation, which results in mating and formation of diploid cells. Since it is homothallic, in genetic crossing for new strain construction, complementary markers that allow for selective growth of crossed diploids is essential (Cregg et al., 2009). All *P. pastoris* expression strains are derived from the wild type strain NRRL-Y 11430. Auxotrophic mutants (e.g., GS115) and protease-deficient strains (e.g. SMD1163, SMD1165, SMD1168) are commonly used. With respect to methanol utilization, there are three phenotypes for *P. pastoris*, namely Mut^+ (methanol utilization wild-type; intact *AOX*1 and *AOX*2), Mut^S (methanol utilization slow; *AOX*1 interrupted, *AOX*2 intact), and Mut^- (methanol utilization deleted, interrupted *AOX*1 and *AOX*2). However, the inducer methanol, being a fire hazard and a toxic component, is a disadvantage of the commonly used *AOX*1 promoter. Alternative promoters and strategies are discussed in the next section. Moreover, with the completion of the genome sequencing project, and the two publicly available genome browsers (Mattanovich et al., 2009b), engineering and use of the *P. pastoris* expression system will be more powerful than ever.

P. pastoris expression system is patented through Research Corporation Technologies (Tucson, AZ, USA), and the expression kit is available for research use from Invitrogen Corporation (Carlsbad, CA, USA). The first therapeutic polypeptide expressed by *P. pastoris*, 60-amino acid ecallantide, received approval from FDA in 2009 and is produced by Dyax Corporation (Cambridge, MA) for the treatment of hereditary angioedema.

Another advantage of this recombinant protein expression system is the absence of α-1, 3-linked mannosyl transferase, which produces the highly immunogenic terminal α-1, 3-linked mannosyl linkages in *S. cerevisiae*, and the less extensive hyper-mannosylation in *P. pastoris* compared to *S. cerevisiae*. While the recombinant human erythropoietin (EPO) expressed in wild type *P. pastoris* contained mostly 17 mannose residues (Çelik et al., 2007), after extensive research on humanization of the *P. pastoris* glycosylation pathway (Bobrowicz et al., 2004; Callewaert et al., 2001; Choi et al., 2003; Hamilton et al., 2003; Vervecken et al., 2004), fully functional recombinant rat EPO with terminal sialic acid residues was finally attained (Hamilton et al., 2006).

2.3. Hansenula polymorpha (Pichia angusta)

H. polymorpha shares a common pathway with *P. pastoris* to metabolize methanol as a carbon and energy source, but differs in having a nitrate assimilation pathway that is not present in other methylotrophs. In basic research, it is used as a model organism for perixisomal function and biogenesis (van der Klei and Veenhuis, 2002) as well as nitrate assimilation (Siverio, 2002).

A number of products currently on the market have proven the value of the *H. polymorpha* expression system, including a recombinant Hepatitis B vaccine, interferon alpha-2a, hirudin, insulin, and phytase, as well as the food supplements hexose oxidase and lipase, both of which have obtained GRAS notification from FDA. Heterologous gene expression in *H. polymorpha* is linked to strong, tunable promoters derived from genes of the methanol utilization pathway, most commonly from the methanol oxidase (MOX) and formate dehydrogenase (FMD) genes. Unlike the *P. pastoris* expression system, which requires methanol for induction, the *H. polymorpha MOX* promoter is significantly de-repressed under glucose limitation or starvation, allowing for a methanol-free process.

H. polymorpha can reach high cell densities, efficiently secrete proteins with a molecular weight of up to 150 kDa and has one of the highest productivity values ever recorded for a yeast expressed protein, with 13,5 g L^{-1} in phytase production (Mayer et al., 1999). Vectors are stably integrated into the genome of *H. polymorpha* over periods of 800 generations (Kang and Gellissen, 2005), providing a reliable expression process. Since *H. polymorpha* is thermotolerant (up to 49 °C), it is well suited for the production of thermostable enzymes and proteins intended for crystallography studies. Moreover, as in *P. pastoris*, the presence of excessive peroxisomal membrane in *H. polymorpha* provides an excellent target for heterologous production of membrane proteins, fused with an appropriate signal peptide, such as that of Pex3p (Baerends, et al., 2000).

Three parental strains of *H. polymorpha* with independent origins have been isolated. Strain NCYC495 is mostly used for classical genetic techniques, while strains CBS4732 and DL-1 (and their derivatives) are typically used in r-protein productions (Kunze et al., 2009).

2.4. Arxula adeninivorans

A. adeninivorans is a temperature-dependent dimorphic yeast, able to grow as budding cells and also as mycelium (Wartmann et al., 1995a). Mycelial cultures are able to secrete higher concentrations of proteins than budding cell cultures (Wartmann et al., 2000). It is haploid, non-pathogenic, can grow on a range of compounds as the sole source of carbon and energy, including n-alkanes and starch (Wartmann et al., 1995b), thermo-resistant (Wartmann et al., 1995a), and halo-tolerant (Yang et al., 2000). Moreover, *A. adeninivorans* produces and secretes several extracellular enzymes into the culture medium during cultivation, including RNAse, proteases, various glucosidases such as glucoamylase, β-glucosidases, pectinases, xylosidase, acid phosphatases, trehalose, cellobiose, invertase and phytase (Wartmann and Kunze, 2000).

2.5. Kluyveromyces lactis

K. lactis has been used in the food industry for the production of lactase (β-galactosidase) since 1950s, and for heterologous expression of bovine chymosin (rennin) (van den Berg et al., 1990).

A number of aspects have contributed to the popularity of *K. lactis* for r-protein production. These include a strong, inducible promoter *LAC4* that is repressed by glucose in low amounts, the ability to utilize cheap substrates like lactose and whey, its approval as a GRAS strain, the ability to secrete high molecular weight proteins (Wésolowski-Louvel et al., 1996), completion of its genome sequencing (Dujon et al., 2004) and the commercially available protein expression kit from NEB (Colussi and Taron, 2005). While the early literature on this organism is based mostly on the MATα mating strain CBS 2359, the wild-type haploid strain GG799 that is included in the commercial kit is currently a well-accepted host.

2.6. Yarrowia lipolytica

Y. lipolytica has become an increasingly popular system for the expression of heterologous proteins because i) it has the inherent ability to secrete high molecular weight proteins in large amounts; ii) proteins can be secreted via the co-translational translocation pathway similar to higher eukaryotes (Biosramé et al., 1998) and in contrast to the post-translational translocation predominant in *S. cerevisiae*; iii) it does not ferment sugars; iv) its genome is completely sequenced and annotated (Casaregola et al., 2000; Dujon et al., 2004); v) an expression kit is available through Yeastern Company (Taiwan); vi) high cell density fermentation is possible (Kim et al., 2000) and vii) it has been approved for several GRAS industrial processes by the FDA. Moreover, Song and co-workers (2007) have performed the first step in humanization of the glycosylation pathway, deletion of the OCH1 gene, to prevent the hyper-mannosylation of proteins.

2.7. Schizosaccharomyces pombe

S. pombe is a fission yeast and grows preferentially as haploids. It was the sixth eukaryotic organism to have its genome fully sequenced (Wood et al., 2002) and the third eukaryotic organism, after *S. cerevisiae* and *Homo sapiens*, for which the complete proteome is available in UniProtKB/Swiss-Prot. This intense characterization is

due to various important properties it shares with higher eukaryotes such as regulation of cell cycle, transcription initiation, chromosomal organization, RNA splicing and the RNA interference (RNAi) pathways (Olsson and Bjerling, 2011; Rhind et al., 2011). Additionally, the Golgi apparatus is morphologically well-defined, glycoproteins can be galactosylated (Moreno et al., 1985) and the quality control mechanism of glycoprotein folding is closer to mammalian cells than *S. cerevisiae* (Parodi, 1999). For these reasons, *S. pombe* is now considered to be an attractive host for expression of mammalian proteins. Nevertheless, an *S. pombe* expression system that is amenable to industrial applications is currently underdeveloped and is the target of ongoing research (Giga-Hama et al., 2007; Idiris et al., 2010; Kjærulff and Jensen, 2005).

3. Vector design

A typical expression vector is composed of a eukaryotic transcription unit with a promoter sequence, a single or multiple cloning site (MCS) for insertion of a foreign gene and a transcriptional termination sequence; a eukaryotic selectable marker gene; and an origin of replication that functions in a eukaryotic cell (2 μm plasmid origin, autonomously replicating sequence (ARS), yeast centromeric sequences (CEN) or a combination of ARS and CEN), if it is not an integrative plasmid. Integrative plasmids applied for transformation are incorporated into the yeast genome by homologous recombination. Therefore these plasmids also contain DNA homologous to the target locus. Integrative plasmids are advantageous in many cases, because no selective pressure is required during the r-protein production process, and multiple tandemly repeated copies may be integrated into the genome, as many as 100 copies reported for *H. polymorpha* (Janowicz et al., 1991; Kunze et al., 2009) and 30 copies for *P. pastoris* (Cregg et al., 2009; Scorer et al., 1994). Shuttle vectors are also commonly employed since insertion of the foreign gene into the expression vector is easier to carry out in *Escherichia coli*. These vectors additionally contain an origin of replication that functions in *E. coli* and an *E. coli* selectable marker (such as Amp^r) gene. For extracellular secretion of recombinant proteins, some commercial expression vectors contain a sequence encoding the secretion signal that is in-frame relative to MCS. Moreover, sequences encoding an epitope tag for affinity purification or detection of the r-protein, such as 6xHis, FLAG, HA and c-Myc tags (Brizzard et al., 1994; Cravchik and Matus, 1993; Martzen et al., 1999), are generally included in-frame with the gene of interest.

Choice of the promoter for heterologous gene expression highly affects product yields. A large variety of heterologous and homologous as well as constitutive and inducible yeast promoters are available, yet toxicity of the product should be considered while choosing a promoter. Commonly used promoters are given in Table 1, and the search for more efficient promoters still persists (Hartner et al., 2008; Partow et al., 2010; Stadlmayr et al., 2010). In addition to the promoters native to the host given in Table 1, promoters derived from *S. cerevisiae* are still widely used in other yeast expression systems, such as *PHO5*, *CUP1*, *GAL1*, *GAL7*, *GAL10* and *PGK*.

The choice of the inducer and the induction mechanism plays an equally important role in promoter selection. In *P. pastoris* fermentations, as the inducer methanol is inappropriate for the production of products used in the food industry, glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter has been preferred widely as an alternative to *AOX*1 promoter (Waterham et al., 1997). In *GAP* promoter expression system, the cloned heterologous protein is expressed along with the cell growth. This system requires no washing to

Table 1
Notable promoters for recombinant protein production in yeast.

Strain	Promoter	Regulation	Reference
S. cerevisiae	*ADH1*	Constitutive	Hitzeman et al. (1981)
	GAPDH	Constitutive	Rosenberg et al. (1990)
	PGK1	Constitutive	Dobson et al. (1982)
	ENO	Constitutive	Holland et al.; (1981)
	PYK1	Constitutive	Burke et al. (1983)
	GAL1–10	Inducible (galactose)	Johnston and Davis (1984)
	ADH2	Inducible (ethanol)	Price et al. (1990)
	CUP1	Inducible (copper)	Karin et al. (1984)
	PHO5	Inducible (phosphate)	Meyhack et al. (1982)
P. pastoris	*AOX1*	Inducible (methanol)	Tschopp et al. (1987)
	FLD1	Inducible (methanol, methylamine)	Shen et al. (1998)
	GAP	Constitutive	Waterham et al. (1997)
	PEX8	Inducible (methanol, oleate)	Johnson et al. (1999)
	YPT1	Constitutive	Sears et al. (1998)
H. polymorpha	*MOX*	Inducible (methanol)	Gellissen (2000)
	FMD	Inducible (methanol)	Gellissen (2000)
	TPS1	Constitutive	Amuel et al. (2000)
K. lactis	*LAC4*	Inducible (lactose)	Van den Berg et al. (1990)
	ADH4	Inducible (ethanol)	Saliola et al. (1999)
	PGK	Constitutive	Rocha et al. (1996)
Y. lipolytica	*LEU2*	Inducible (leucine precursor)	
	RPS7	Constitutive	Müller et al. (1998)
	XRP2	Inducible (peptones)	Ogrydziak and Scharf (1982)
	TEF	Constitutive	Müller et al. (1998)
S. pombe	$adh1^+$	Constitutive	Russell and Hall (1983)
	SV40	Constitutive	Kaufer et al. (1985)
	CaMV	Inducible (tetracycline)	Forsburg (1993)
	$ctr4^+$	Repressible (copper)	Bellemare et al. (2001)
	nmt1	Repressible (thiamine)	Basi et al. (1993)
	$invl^+$	Repressible (glucose)	Iacovoni et al. (1999)
	pho1	Repressible (adenine)	Schweingruber et al. (1992)
A. adeninivorans	*TEF1*	Constitutive	Wartmann et al. (2002)
	AHSB4m	Constitutive	Wartmann et al. (2003)
	GAA	Inducible	Bui et al. (1996)
	AINV	Inducible	Böer et al. (2004a)
	AXDH	Inducible	Böer et al. (2004b)

remove the non-methanol carbon sources, thus, features of the GAP expression system may contribute significantly to the development of cost-effective methods for large-scale production of heterologous r-proteins (Cos et al., 2006). The combination of *AOX*1 and *GAP* promoters to co-express recombinant proteins has been developed (Wu et al., 2003), in order to increase the expression levels of *GAP* promoter. The idea is to induce *AOX*1 promoter with methanol after the exhaustion of glucose. The secreted protein concentration was about two-fold higher compared to *GAP* promoter alone, with appreciable differences in cell concentration obtained (Wu et al., 2003).

Introduction of the expression vectors into the yeast cells, transformation, can be mediated by electroporation, lithium acetate or lithium chloride treatment, and cell wall removal (spheroplast preparation). Electroporation for its ease and lithium acetate treatment for its cost efficiency are the currently preferred methods.

A selective marker is necessary not only to select for transformants but also to ensure plasmid survival through generations. Dominant selection markers include genes conferring resistance to copper (Fogel and Welch, 1982), and to particular antibiotics, such as chloramphenicol (Jimenez and Davies, 1980), G418 (Webster and Dickson, 1983), hygromycin (Gritz and Davies, 1983) and zeocin (Pfeifer et al., 1997). The use of antibiotics may be expensive, undesired in food and therapeutic products and the antibiotic may be degraded or inactivated, rendering this approach ineffective in certain situations. Nevertheless, it does not require host strain engineering and can be used for selection in rich cultivation medium. On the other hand, auxotrophic selection markers (including *HIS3*, *HIS4*, *LEU2*, *LYS2*, *TRP1*, *URA3*) are designed to complement a specific auxotrophic mutation in the host strain. However, as these mutations are recessive, a suitable recipient is difficult to obtain when dealing with industrial yeast strains. For *S. cerevisiae* and *K. lactis*, the *LEU2* gene and the G418 resistance gene are the most popular markers, whereas for *H. polymorpha*, *S. pombe* and *Y. lipolytica* the *LEU2* and *URA3* genes are mainly used, and for *P. pastoris*, *HIS4* and zeocin resistance genes are commonly employed. The selective marker can be rescued using the Cre–*loxP* system and reused, an approach first used in *S. cerevisiae* (Guldener et al., 1996; Sauer, 1987), then used in several other yeast, *K. lactis* (Steensma and Ter Linde, 2001), *S. pombe* (Iwaki and Takegawa, 2004), *Y. lipolytica* (Fickers et al., 2003) and *P. pastoris* (Pan et al., 2011).

A wide-range yeast vector system (CoMed™) provides a versatile tool to address a range of yeasts, namely *H. polymorpha*, *A. adeninivorans*, *S. cerevisiae*, *P. pastoris* and *K. lactis*, simultaneously with a single vector (Steinborn et al., 2006). In its basic form, the vector is composed of genetic modules that are functional in all yeasts, namely a rDNA targeting sequence, an appropriate selection marker and an expression cassette under control of a *TEF*1 promoter from various sources.

4. Fermentation process optimization

The bioprocess operation parameters, including the culture medium composition, temperature, pH, agitation and aeration rates, induction and feeding strategies can highly influence the product yield and quality. This section provides an overview of process optimization examples, excluding the well-established *S. cerevisiae* expression system.

4.1. Pichia pastoris

For production of recombinant proteins in high cell density fermentation processes, the most commonly used medium is composed of a basal salt medium, trace salts solution (PTM1), ammonium hydroxide as the nitrogen source, where the hydroxyl groups in ammonium hydroxide also serve to maintain the pH, and various combinations of glycerol/methanol feed as the carbon and energy source. While the *AOX*1 promoter in Mut$^+$ and Mut^s strains is induced by methanol, cell growth is inhibited above 4 g L^{-1} methanol concentration (Zhang et al., 2000). To keep methanol concentration below the toxic limit, fed-batch feeding strategies with a three or four-stage feeding process is commonly employed (Sinha et al., 2004; Stratton et al., 1998; Zhang et al., 2000). In a three-stage process, first, the engineered strain is cultured in medium containing glycerol, a non-fermentable but repressing carbon source, in order to accumulate cells. Second, a glycerol fed-batch transition phase is applied at limiting glycerol levels to further increase cell concentration without repressing growth. Another purpose of the second stage is to prepare the cells for another carbon source. In the third stage, methanol is added to the fermentation medium in fed-batch mode, starting the induction phase. In a four-stage process, an additional stage of batch-methanol addition is employed in between the 2nd and 3rd stages (transition phase), to prepare the cells prior to fed-batch operation.

The use of glycerol as the co-substrate is one of the most common strategies (Jungo et al., 2007a; Orman et al., 2009; Zhang et al., 2003). However, Xie et al. (2005) presented that the excess glycerol in the medium represses the *AOX*1 promoter, i.e. protein production. As an alternative to glycerol/methanol co-feeding strategy, many other studies were conducted with different feeding strategies, especially using sorbitol as co-substrate, a non-repressing carbon source (Çalık et al., 2010a; Çelik et al., 2009a; Jungo et al., 2007b; Wang et al., 2010). While Jungo et al. (2007b) used a fed-batch feeding strategy using 43% methanol and 57% sorbitol (C-mol C-mol^{-1}) under two different feeding rates, Çelik et al. (2009a) showed that below 50 g L^{-1} sorbitol did not repress growth or production, and hence added 50 g L^{-1} sorbitol batch-wise in the beginning of the production phase. Moreover, the crude glycerol by-product of the ever-growing biodiesel industry, with methanol contaminant that limits its further use in the industry, was shown to be a highly suitable and inexpensive substrate for the methylotrophic yeast processes (Çelik et al., 2008).

Specific growth rates on methanol were shown to influence r-protein production (Çelik et al., 2009a; Kobayashi et al., 2000; Zhang et al., 2005). Jungo et al. (2007b) indicated that maximum specific growth rate on methanol was $\mu_{max} = 0.14\ h^{-1}$, and conducted two fed-batch bioreactor experiments at $\mu_o = 0.03\ h^{-1}$ and $\mu_o = 0.05\ h^{-1}$ using 43% methanol and 57% sorbitol (C-mol C-mol^{-1}) for recombinant avidin production and pointed out that for μ values larger than 0.02 h^{-1}, productivity increases slightly with μ. It was also shown that above $\mu = 0.032\ h^{-1}$ dual carbon source limitations would occur. Moreover, in a more recent study, Çelik et al. (2009a) investigated the effect of batch-wise sorbitol addition in the fed-batch production phase for glycosylated protein, human erythropoietin, at different methanol feeding rates, and obtained slightly higher product yield at $\mu_o = 0.03\ h^{-1}$, where the highest cell concentration was achieved at $\mu_o = 0.02\ h^{-1}$ in the presence of co-substrate sorbitol. This strategy was also successfully applied for human growth hormone production (Çalık et al., 2010a).

Because the oxygen is used in oxidation of methanol to formaldehyde as a side-reaction, higher oxygen transfer rates are required in the methanol utilization pathway, which leads to product formation (Sibirny et al., 1990). To provide the required amount of oxygen, transfer resistances are minimized by high agitation, and oxygen enriched air is used. In most of the *P. pastoris* fermentation processes, the dissolved oxygen is kept around 20–30% (Çelik et al., 2009a; Horstkotte et al., 2008; Surribas et al., 2007; Thorpe et al., 1999; Xie et al., 2005).

Most processes with *P. pastoris* are run at a temperature of 30 °C, which is optimum for growth. By decreasing growth temperature from 30 to 20 °C, a 3-fold increase in specific r-protein productivity was obtained in chemostat cultures of *P. pastoris*, which was accompanied by a reduced flux through the TCA-cycle, reduced levels of proteins involved in oxidative stress response and lower cellular levels of molecular chaperones (Dragosits et al., 2009).

The medium pH plays an important role in fermentation processes, since cell growth rate, enzyme activity, and proteolytic degradation are dependent on the culture pH. Although pH values within 3.5 to 5.5 were reported to have little effect on the growth rate for *P. pastoris*

Mut$^+$ strain (Inan et al., 1999), different pH values were found to be optimal for the production of different proteins (Macauley-Patrick et al., 2005). While for EPO producing r-*P. pastoris*, the highest product formation was obtained with a two stage pH operation strategy where glycerol batch, and glycerol fed-batch phases were operated at pH = 5.0 and methanol induction phase was operated at pH = 4.5 (Soyaslan and Çalık, 2011); for hGH producing r-*P. pastoris*, the highest product formation was obtained at pH = 5.0 (Çalık et al., 2010b).

4.2. Hansenula polymorpha

Process development in *H. polymorpha* was recently reviewed by Stöckmann et al. (2009). Similar to *P. pastoris*, a defined minimal mineral medium composed of salts, vitamins and trace elements is used to support growth. Based on the promoter (*FMD* or *MOX* promoter), fermentation strategies are developed using either glucose or glycerol in the beginning of fermentation, followed by carbon source limitation in the second phase (Hellwig et al., 2005; Stöckmann et al., 2009).

Three different fermentation modes are generally used. In glycerol starvation processes, after consumption of the glycerol, a feed is initiated that adds glycerol by a pO2-controlled feeding device followed by operation of the bioreactor under glycerol limited conditions (Müller et al., 2002; Zurek et al., 1996). For glucose starvation processes, only glucose is used as the carbon source. After utilization of glucose initially fed to the bioreactor, a glucose-limiting feed is initiated that adds the carbon source with a stepwise increase in feeding rate, in correlation to the cell mass (Mayer et al., 1999). In a third type of process, the batch phase and the first fed-batch phase is similar to the glycerol starvation processes, but followed by a mixture of glycerol and methanol feed added during the last hours of the fermentation (Melmer et al., 2008). In a more recent study, secondary substrate-limited batch fermentation was shown to divert metabolic flux towards protein production and increase GFP production by 1.9-fold (Kottmeier et al., 2010).

H. polymorpha was used for the production of recombinant proteins, i.e., IFNα-2a (Müller et al., 2002), interleukin-6 (Böer et al., 2007), recombinant human serum albumin (Heo et al., 2008; Kang et al., 2001; Morawski et al., 2001) and enzymes, i.e., glucose oxidase (Hodgkins et al., 1993) glycolate oxidase and catalase (Gellissen et al., 1996), phytase (Mayer et al., 1999), isopenicillin-N synthase (Gidijala et al., 2008). Additionally, *H. polymorpha* was used as the host for overexpression of bacterial xylose isomerase for alcoholic fermentation (Dmytruk et al., 2008).

4.3. Arxula adeninivorans

A dimorphic species *A. adeninivorans* can utilize adenine, xanthine, uric acid, putrescine and *n*-alkylamines as carbon, nitrogen or energy sources in addition to glucose (Böer et al., 2005, 2007). No industrial processes have been developed so far based on *A. adeninivorans* (Stöckmann et al., 2009). Nevertheless, Böer et al. (2011) recently reported large scale tannase production using various plasmids carrying one or two expression modules for constitutive expression of tannase, where they obtained 51,900 U of tannase activity L^{-1} and 162 g L^{-1} cell concentration at t = 142 h of the fed-batch process.

4.4. Kluyveromyces lactis

Donnini et al. (2004) reported that by using a glucose repression-defective mutant of *K. lactis*, they have improved the production of heterologous proteins, i.e., glucoamylase, human serum albumin, and human interleukin-1 compared to the parent strain. Camattari et al. (2007) reported the production of human interleukin-1β using a hypoxic induction strategy. Moreover, Raimondi et al. (2010) investigated the effects of medium composition on superoxide dismutase production in *K. lactis*.

K. lactis was successfully used for the production of recombinant proteins, i.e., interleukin-1β (Blondeau et al., 1994a; Fleer et al., 1991a), granulocyte colony-stimulating factor (Hua et al., 1994), hepatitis B surface antigen (Martinez et al., 1992) and human serum albumin (Bao and Fukuhara, 2001; Blondeau et al., 1994b; Fleer et al., 1991b; Lodi et al., 2005; Saliola et al., 1999), and enzymes, i.e., prochymosin (Van den Berg et al., 1990), β-galactosidase (Bergkamp et al., 1992; Panuwatsuk and Da Silva, 2003), D-amino acid oxidase (Gonzalez et al., 1997), glucoamylase (Merico et al., 2004), lipase (Müller et al., 1998), xylanases (Walsh and Bergquist, 1997; Walsh et al., 1998), and esterase (Rocha et al., 2011).

4.5. Yarrowia lipolytica

Although extensive data on *Y. lipolytica* cultivation was reported in the literature, these reports mostly describe the production of citric acid, single cell proteins or cognate proteins like lipases, and may not be fully adapted to recombinant protein production (Fickers et al., 2004; Gasmi et al., 2011; Madzak et al., 2004; Turki et al., 2010,). Furthermore, studies on the expression of heterologous genes in this yeast rely on the use of complex media and shake-flask cultures (Gasmi et al., 2011). Medium composition and production strategies for *Y. lipolytica* have not been extensively studied; an in-depth analysis of fermentation characteristics and strategies are required.

4.6. Schizosaccharomyces pombe

To use *S. pombe* as an efficient host for heterologous protein production, the minimal genome was attempted by deleting as many non-essential genes as possible (Giga-Hama et al., 2007). The reasoning behind this was that many *S. pombe* genes are unnecessary under nutrient-rich growth conditions, and wastes energy from the viewpoint of heterologous protein production. Moreover, *S. pombe* strains defective in up to seven proteases have been created (Idiris et al., 2006), in order to increase heterologous protein production.

S. pombe was used for the production of human lipocortin I (Giga-Hama et al., 1994), D-amino acid oxidase of rigonopsis (Isoai et al., 2002), Cytochrome P450 enzymes (Bureik et al., 2002; Zöllner et al., 2009), human papillomavirus-type-16 vaccine (Sasagawa et al., 2005), *E. coli* phytase (Ciofalo et al., 2003), human lysosomal acid lipase (Ikeda et al., 2004), human growth hormone (Idiris et al., 2006), human transferrin (Ohashi et al., 2010), fluorescent single-chain Fv (scFv) antibody fragments (Naumann et al., 2011a), and 20-α-dihydrodydrogesterone (Naumann et al., 2011b).

5. Post-translational modifications and secretion

Yeast have the ability to perform many of the post-translational modifications similar to those performed in higher eukaryotes, such as correct folding, disulphide bond formation, *N*- and *O*-linked glycosylation and proteolytic processing of signal sequences. In some cases, folding and disulphide bond formation have been identified as the 'rate-limiting' steps in the production of foreign proteins (Hohenblum et al., 2004), thus the ability of the organism to process, fold and secrete the recombinant products determines the productivity of the expression system. In order to obtain high yields of functional proteins, these rate-limiting factors are addressed by optimization of the fermentation process and most effectively by strain engineering using genetic modifications, post-genomic technology and systems biology tools.

Protein folding is a complex process utilizing molecular chaperones (e.g., BiP, calnexin, and calreticulin) that assist protein folding and foldases such as protein disulfide isomerase (PDI) and *cis–trans* peptidyl prolyl isomerase (PPI). The comprehensive characterization of genes required for endogenous protein folding has been described (Jonikas et al., 2009). Removal of misfolded proteins through

endoplasmic reticulum (ER) associated degradation (ERAD) occurs by ubiquitination via ER-associated ubiquitin-conjugating enzymes, followed by retrotranslocation, and degradation in the cytoplasm by the proteasome (reviewed in Nakatsukasa and Brodsky, 2008). The accumulation of ERAD substrates leads to the induction of unfolded protein response (UPR), recently reviewed in detail (Patil and Walter, 2001; Stolz and Wolf, 2010). Numerous engineering strategies have targeted this protein folding and quality control system to increase secretion of heterologous proteins in yeast (reviewed by Idiris et al., 2010); examples include overexpression of BiP or PDI (Shusta et al., 1998), co-overexpression of BiP and PDI (Damasceno et al., 2006), and constitutive expression of the UPR transcription factor (TF), Hac1 (Valkonen et al., 2003).

Although cytoplasmic expression often leads to high expression levels in yeast, secretion of proteins to the extracellular medium is preferred to simplify the downstream processes. The *S. cerevisiae* α-mating factor (α-MF) pre–pro leader sequence is the most widely used secretion signal, being better than the native leader sequence of the heterologous protein in some cases. This signal peptide has also been engineered further (MF α1pp), increasing human IgG1 production levels up to 180-fold over wild type (Rakestraw et al., 2009). Other common examples include signal sequences of *S. cerevisiae* invertase (SUC2), *P. pastoris* acid phosphatase (PHO1), *Y. lipolytica* alkaline extracellular protease (XRP2) and *K. lactis* presequence of the α-subunit of the K1 killer toxin. However, not only the sequence of the N-terminal signal peptide but also the properties of the signal–anchor sequences determine whether a protein follows the co- or post-translational pathway (Berndt et al., 2009; Kida et al., 2009). Therefore, different approaches (recently reviewed by Idiris et al., 2010), such as fusion expression with other protein molecules or subdomains may improve results. While several fusion partners are available for bacterial expression to improve secretion, relatively few exist for yeast; recent examples including CBM21 (carbohydrate binding module family 21)-starch binding domain (Lin et al., 2009), *E. coli* MBP (maltose binding protein) (Dalken et al., 2010; Li et al., 2010), bacterial Hsp70 (heat shock protein) (Yang et al., 2009) in *P. pastoris*, and Pir4 (protein with internal repeats) in *S. cerevisiae* (Paciello et al., 2010).

Glycosylation is the most abundant protein modification occurring across all kingdoms of life (Larkin and Imperiali, 2011), and may influence the solubility, folding, susceptibility to proteolysis, thermal stability, receptor binding and *in vivo* activity of the protein (Dixon, 1991; Yamaguchi et al., 1991). Yeast are capable of performing both *N*- and *O*-linked glycosylation, however there are many differences in glycosylation between yeast and mammalian cells. The *N*-linked glycosylation pathway in the ER is conserved between yeast and most higher eukaryotes, and involves the formation of a Glc3Man9GlcNAc2-PP-dolichol lipid-linked precursor, the glycan portion of which is transferred co-translationally in the ER to suitable asparagine (Asn) residues on nascent polypeptides (Gemmill and Trimble, 1999). Subsequently, glycohydrolases in the ER remove the three glucoses and (with the exception of *S. pombe* and *K. lactis*) a specific mannose residue.

N-Glycan processing pathways are greatly diverse among species after transportation of predominantly Man8GlcNAc2-containing glycoproteins to the Golgi apparatus and have been reviewed before (Gemmill and Trimble, 1999). *S. cerevisiae* has the most extensive hyper-glycosylation, typically adding 50–150 mannose residues, while *Y. lipolytica* was reported to add 8–10 residues (Madzak et al., 2004) and *P. pastoris* and *H. polymorpha* add up to 20 residues in total. Terminal mannose residues in *N*-linked glycans are added in *S. cerevisiae* by an α-1,3 bond which is considered to be allergenic; whereas in *H. polymorpha* and *P. pastoris*, a nonallergenic α-1,2 bond is present (Jigami and Odani, 1999). *S. pombe*, having galactosyltransferase activity, N-terminal acetylation and polyisoprenylation capabilities, has more similar post-translational modifications to mammalian cells compared to other yeast species. In *A. adeninivorans*, *O*-glycosylation was found to be restricted to the budding yeast status, in the case of AFet3p (Wartmann et al., 2002).

As about 70% of all therapeutic proteins are glycoproteins, correct glycosylation of proteins is critical in therapeutic efficacy of the protein because non-human glycosylation can reduce the half-life of the protein and elicit an immunogenic response to the foreign carbohydrate moiety (Helenius and Aebi, 2001). Thus, glycoengineering of yeasts have been remarkable in the last two decades. Studies on humanization of the *N*-glycosylation pathway (recently reviewed in Chiba and Akeboshi, 2009; De Pourcq et al., 2010) focused on deleting the yeast genes involved in hyper-mannosylation initiated by Och1p and the species-specific genes responsible for the modification of sugar chains at the non-reducing end. These steps were followed by the introduction of genes catalyzing the synthesis, transport, and transfer of human sugars, finally achieving the production of sialylated erythropoietin in the glycoengineered *P. pastoris* strain (Hamilton et al., 2006). Glycoengineering studies with the *H. polymorpha* have yielded glycoproteins mainly containing trimannosyl core *N*-glycan ($Man_3GlcNAc_2$) (Oh et al., 2008).

6. Conclusions and future perspectives

Various yeast expression systems have been successfully used over the years for production of recombinant proteins including therapeutic proteins. The availability of large strain collections, wide variety of vectors, promoters, dominant or auxotrophic markers and efficient transformation protocols, combined with the long tradition of large-scale fermentation techniques, as well as the revolutionary advances in the next-generation DNA sequencing, systems and synthetic biology, micro-array technology and the rapidly evolving databases on the Web, has secured the yeast a prominent place within the range of expression platforms.

Efforts to humanize the glycosylation pathway similar to that in *P. pastoris* and to create a homogenous glycan on the target protein in yeast will continue in other yeast hosts, since further development of glycoengineered yeast systems will lead to the production of lower-priced pharmaceutical products. Additional studies are also required to master the quality control mechanism in ER.

Still more profound comprehension of cell physiology and stress response to protein production would favor higher yields and higher quality protein production. In this respect, the yeast deletion collections for *S. cerevisiae* (Giaever et al., 2002) and *S. pombe* (Kim et al., 2010) creates new opportunities for genome scale comparative studies and identifying novel pathways that can be targeted for metabolic engineering of host strains.

Comprehensive mathematical models of the yeast metabolism are also becoming popular to identify or simulate metabolic engineering strategies that may substantially improve the process of cell factory development (Papini et al., 2010). The type of the model to use depends on the objective of the study as well as the available data. While genome-scale models are currently only available for *S. cerevisiae* (Herrgard et al., 2008) and *P. pastoris* (Sohn et al., 2010), smaller scale stoichiometric and kinetic models in various yeast species notably elucidate the impact of bioprocess operation parameters on recombinant protein production (Çelik et al., 2009b, 2010; Dragosits et al., 2009; Herwig and von Stockar, 2003; Papanikolaou et al., 2006; Sola et al., 2007).

In addition to being industrial hosts for r-protein and metabolite production, yeast are used as a powerful tool in high-throughput assays for functional genomics and drug screening. The highly efficient homologous recombination feature of yeast is widely exploited as a genetic engineering tool to overcome the difficulties in cloning larger segments of DNA; even in creating the synthetic bacterium recently (Gibson et al., 2008; Lartigue et al., 2009), opening the doors to a new era.

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