PICHIA, OPTIMIZATION OF PROTEIN EXPRESSION

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INTRODUCTION

A wide variety of expression systems for production of proteins of interest have become available with the advent of recombinant DNA technology since the 1970s. The various expression systems include those based on bacteria, lower eukaryotes (yeasts and fungi), invertebrates (insect cells and larvae), vertebrates (cells and transgenic animals), and plants (cells and transgenic plants). Each of these systems has unique advantages. Yeasts and fungi have robust growth characteristics like bacteria. They are also readily amenable to genetic manipulation. Furthermore, they can perform many posttranslational modifications found in higher eukaryotes. The purpose of this article is to highlight the strategies that have been used for optimal protein expression with the *Pichia* yeast expression system, which in the past few years has turned out to be versatile and impressive yeast for production of proteins.

BACKGROUND

Pichia pastoris is a methylotrophic yeast. It is able to use methanol as sole carbon source for energy as well as for growth (1,2). It was originally developed by Phillips Petroleum Company (Bartlesville, Oklahoma) as an organism of choice for bioconversion of natural gas (methane) into food (single cell protein). This may sound ironic, because nowadays every one is talking bioconversion of food crops to fuels (biofuels). Phillips Petroleum Company in the 1970s developed an efficient *Pichia* fermentation process [cell density >130 g dry cell weight per liter and biomass productivity >10 g/(L h)] (3,4). Though impressive, it could not compete with the economics of production of soy protein. After this set back, Phillips in the early 1980s directed its future course with *Pichia* into two areas:

1. *Speciality Food or Feed Application:* A rather impressive 100,000 L fermentation plant to churn out tons of *Pichia* for potential speciality food applications was completed in 1988. However, by 1993, the idea to use *Pichia* for speciality food/feed was abandoned as *Pichia* did not have generally regarded as safe (GRAS) status and Phillips Petroleum Company decided to focus on its core business of oil exploration, production, and petrochemicals.
2. *Development of Pichia Expression System for Production of High Value Recombinant Proteins: Pichia* possesses a highly regulated pathway for the utilization of methanol (2,4). Synthesis of the enzymes of methanol metabolism are undetectable in the absence of methanol, but increase rapidly when methanol is used as the sole carbon source. The most dramatic effect is seen for alcohol oxidase (AOX1), which accounts for >30% of cellular protein (5). An extensive proliferation of the peroxisomes, known to sequester AOX1 and dihydroxy acetone synthase, is also observed in cells grown on methanol (5–7). Perhaps the decision of Phillips to exploit the above noted unique features of *Pichia* to develop a protein expression system was highly fortuitous, because the molecular genetic tools that had just become available for *Saccharomyces cerevisiae*, such as yeast–*E.coli* shuttle vectors, transformation protocols, and site-specific integrative transformation (8,9) were found to be readily applicable to *Pichia*. With the advent of cloning of *AOX1* promoter (10,11), availability of auxotrophic *Pichia* strain [GS115 (*his4*) developed by George Sperl] and *Pichia* transformation protocols (12,13), successful high level expression of several proteins was readily demonstrated. They include, intracellular expression of Hepatitis B surface antigen particles (14), *E. coli* β-galactosidase (LACZ) (15), human tumor necrosis factor (TNF) (16), as well as greater than gram per liter level of secretion of *S. cervesiae* invertase (SUC2) (17), and human serum albumin (HSA) (18). *Pichia* expression technology patent was granted to Phillips Petroleum Company in 1988 (11) and thus, by the late 1980s, *Pichia* expression system was well on its way to leave the nest.

In the fall of 1988, Phillips Petroleum Company made a conscientious decision to license the system to other companies. Fortunately for Phillips licensing team (Katherine Bartosh, L.V. Benningfield, Mary Jane Hagenson, Jack Phillips, and Koti Sreekrishna), their very first commercial licensee (Glaxo–Wellcome) with just 2 days of training at Phillips produced some impressive expression results (19–23). Phillips was able to license the technology to 20 companies in 4 years. Along side, it also started distributing the *Pichia* expression kit, free of cost to any academic institution that approached the company for the kit. This was getting out of hand, and thus the company transferred distribution rights, free of cost, to Invitrogen Corporation (Carlsbad, California) in 1993. Since then, Invitrogen Corporation (http://www.invitrogen.com) has been aggressively distributing *Pichia* expression kit at reasonable cost and with some user friendly modifications. They are largely responsible for wide usage of *Pichia* technology. In the same year (1993), Phillips sold *Pichia* technology to Research Technology Corporation (Tucson,

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Arizona) (http://www.rctech.com). They have licensed the technology thus far to 160 companies. To date, >500 proteins have been expressed in the *Pichia* yeast (24) and over 3000 scientific articles published. There are over 100 publications on fermentation optimization alone.

Hepatitis B vaccine and interferon-α derived from *P. pastoris* have been commercialized in India since 1999 and 2002, respectively, by Shantha Biotechnics (http://www.shanthabiotech.com) (25). Now the company makes >100 million units of hepatitis vaccine per year and sells these vaccines in 50 countries. The cost of vaccine has dropped to 15 cents a dose (24). Recombinant human insulin produced in *Pichia* is also marketed in India since 2003, by joint venture between Shantha Biotechnics and Biocon (http://www.biocon.com) (24,25).

HSA expression technology originally demonstrated at Phillips Petroleum Company (18) and transferred to Green-Cross Corporation (Osaka, Japan) has been further developed and scaled up to produce one million dosage vials (12.5 g per vial) of authentic rHSA/year by Mitsubishi Pharma Corporation (Osaka, Japan) (26,27). This truly is a testimony to the high expression level and scale-up possible with *Pichia* (25). (Please refer to http://www.rctech.com for a more complete list of *Pichia*-produced products under various stages of commercialization.)

STRATEGIES FOR OPTIMIZATION OF PROTEIN EXPRESSION

The typical *Pichia* expression vectors are all yeast–*E. coli* shuttle plasmids (see "Glossary of *P. pastoris* Vectors"). The most commonly used vectors are based on *AOX1* promoter (10,11) (see section titled "Alternative Promoters for Expression" below for other promoters available for *Pichia* expression). Numerous selectable marker genes available include *HIS4* (His^+ selection) (12), *ARG4* (Arg^+ selection) (28), A*DE1* (Ade^+ selection) (29), and *URA3* and *URA5* (Ura^+ selection) (29,30), which can be used in conjunction with the appropriate *Pichia* auxotrophic strains. Several dominant selection markers that can be used for transformation of any *P. pastoris* strain are also available. These include *SUC2* (allows growth on sucrose) (13), Kan^R (G418/Geneticin selection) (23,31), Zeo^R (Zeocin selection) (32), Bsd^R (Blasticidin selection) (http://www.invitrogen.com), FLD1 (Formaldehyde selection) (33), and Sor^R (Soraphen A selection) (34).

A wide assortment of proteins have been produced in *Pichia*, which include human insulin (35), glucagon-like peptide (GLP) (36), G-protein coupled receptors (GPCRs) (37), Aquaporin (AQP1) (38), as well as correctly assembled human collagen fibers (39) and fibrinogen chains (40). These are among >500 proteins that have expressed in this system (24). Obviously, not all proteins are expressed at multiple grams per liter range. The expression level is largely influenced by inherent properties of a protein. Some proteins are readily expressed at high levels with minimal manipulation, while some proteins barely reach milligram levels, despite tremendous effort. Encouragingly, in many instances, the initial production yield of a protein can be dramatically enhanced by addressing the multiple factors that influence protein expression (41–43). The purpose of this review is to highlight the various expression-optimization strategies.

Cellular State of the Expression Cassette

The expression cassette can be introduced into *Pichia* by way of chromosomal integration or autonomous replication. Chromosomal integration is preferable because of the following advantages: (i) stability of expression cassette; (ii) ability to generate clones that stably maintain multiple copies of the expression cassette (see section titled "Gene Dosage: Exploiting the Clonal Variation of Expression"); (iii) control over the site of integration (for example, *AOX1*, *HIS4*, *ARG4*, *URA3* loci); and (d) ability to engineer different modes of integration (with or without eviction of the *AOX1* coding sequences) (see section titled "Methanol Utilization Phenotype of the Host: Mut^+ or Mut^s").

Plasmids based on autonomous replication sequence (*ARS*) such as pHIL-A1 (see "Glossary of *P. pastoris* Vectors") although can be introduced into *Pichia* cells with a high transformation frequency, they are rapidly lost from the population of dividing cells, and eventually integrate at one or more of the homologous sites on the chromosome (12,13). Owing to their ability of transform *Pichia* at high frequency ($>10^5$ μg^{-1}) and ease of plasmid rescue, the autonomous plasmids are useful for cloning genes in *Pichia* by functional complementation.

Site of Integration of the Expression Cassette

The *AOX1* promoter used in *Pichia* expression vectors is active irrespective of the site of integration (*AOX1*, *HIS4*, *ARG4*, *ADE1*, or *URA3* loci). However, AOX1 locus is the preferred site of integration for stable expression, because integration at the other loci can result in loss of the expression cassette due to intrachromosmal cross over between the mutant and good copy of the gene (unpublished observations). If the vector also contains a dominant selection marker (discussed earlier), then selection pressure can be applied for stable maintenance of expression cassette at those sites.

Methanol Utilization Phenotype of the Host: Mut^+ or Mut^s

Transformation of a *P. pastoris his4* strain (GS115) using linear DNA expression cassette with the ends bearing homology to the 5′ and 3′ regions of the *AOX1* chromosomal locus results in the site-specific eviction of the *AOX1* structural gene at a high frequency (5–20% of the His^+ transformants) (16,28). Such clones can be readily distinguished by replica-plating the colonies from the initial His^+ selection plate on to a minimal methanol (MM) medium plate. The clones that have undergone eviction of *AOX1* grow slower (Mut^S) compared to Mut^+ clones with an intact AOX1 gene, because, in such clones, growth on methanol is dependent on the alcohol oxidase encoded by *AOX2*, which is expressed at much lower level (weaker promoter) (44). The Mut^+ clones arise due to circularization of the linear DNA expression cassette inside the yeast cell prior to integration. Thus, both Mut^S and Mut^+ clones result in the same experiment.

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Alternatively, one can use the *Pichia* strain KM71 (*his4 arg4 aox1*Δ:*SARG*), which has been rendered Mut^S by replacing much of the chromosomal *AOX1* with *S. cerevisiae ARG4* (28).

For intracellular expression, it is critical to use Mut^S cells because they will have a lower level of *AOX1* and the expressed protein of interest can be more readily purified. Furthermore, the precious cellular machinery can be more fully utilized for the expression of protein of interest. For protein secretion, the choice of Mut^+ or Mut^S is less stringent and more a matter of preference as the impact is more on feed protocols than the secretion level. In fact, the secretion levels of HSA are similar with both Mut^+ and Mut^S cells (45).

Gene Dosage: Exploiting the Clonal Variation of Expression

Early on a dogma prevailed among the researchers of the *Pichia* system, based on limited experience (14,15), that increasing the gene dosage did not impact expression because of the remarkable strength of the *AOX1* promoter. This dogma was shattered on the Good Friday of March 28, 1986, with my observation of dramatic clonal variation (<1% to >30%) in the expression of human TNF, that was subsequently attributed to gene dosage. Furthermore, the high copy number was stably maintained even after several days of growth in high cell density fermentor. The observation with TNF expression was readily exploited for expression of tetanus toxin fragment C (21), *Bordetella pertussus* pertactin P69 (20), and mouse epidermal growth factor (EGF) (19), and has been a key strategy for successful expression of hundreds of proteins in the *Pichia* system. However, in numerous cases, a single copy of the expression cassette is sufficient and deliberately increasing the copy number had no significant effect on the production (14,15,17,18). In some rare instances, an increase in copy number has a deleterious effect on the production level as has been noted with secretion of human insulin-like growth factor (IGF) (46) and *Necator americanus* (hookworm) secretory protein (Na-ASP1) (47).

Because the effect of gene copy number on expression is unpredictable, it is prudent to examine the production level as a function of gene dosage. The spheroplast method of transformation of *Pichia* results in transformants with a wide range of copy numbers (16,48). Evaluation of as few as 100 individual clones for protein production is generally adequate to arrive at a good producer. Though bit laborious, spheroplast method of transformation yields clones with wide range of gene dosage at a high frequency (48). If other methods of transformation such as using LiCl or electroporation, which do not yield high frequency of multicopy clones, then one can use more efficient screens. These include use of colony hybridization with DNA probes or using appropriate vectors that would allow selection based on increased level of resistance to one of G418/geneticin (23,31), zeocin (32), formaldehyde (33), blasticidin (http://www.invitrogen.com), or soraphen A (34). Increased drug resistance does not automatically ensure multicopy integration and several dozen resistant colonies must be analyzed for copy number and expression.

Another approach to identify high producers may be to use a visual tag to the protein being expressed. In one instance, C-terminal green fluorescent protein fusion was used as a visual reporter to identify human Mu-opioid receptor expressing clones (49). *Rhizopus oryzae* lipase (ROL) bearing an N-terminal GFP tag was more efficiently secreted in *Pichia* compared to one with a C-terminal GFP tag (50). Recently, a fusion protein ZZ-EGFP consisting of ZZ domain of staphylococcal protein A (SpA) with enhanced green fluorescent protein (EGFP) was also secreted in the *Pichia*, with a hexahistidine tag (51). GFP tag may serve as a visual reporter to readily identify high producing clones. It can also be useful for on-line monitoring of protein production in the fermentor (52) However, on-line monitoring can be complicated by high levels of riboflavin (excitation/emission = 450/530 nm) secreted by *Pichia* (50), which in itself has been used for monitoring biomass production (53). By using a fluorescent protein tag outside the interfering range, one may be able to reliably monitor simultaneously both cell and product yield on-line.

Vectors such as pAO815 (54,55) have also been described that would allow *in vitro* construction of expression cassette concatamers. This approach is useful to accurately correlate copy number to expression level over a narrow range of gene dosage.

Translational Optimization: 5′ Untranslated Region

The nucleotide sequence and the length of the 5′ untranslated region (5′UTR) are detrimental to optimal protein translation. The leader length of the highly expressed *AOX1* mRNA is 114 nucleotides long, and the sequence is A + U rich (10,11). For optimal synthesis of heterologous proteins, it is essential that the 5′UTR should closely resemble that of the *AOX1* mRNA. Ideally, it is preferable to make it identical to that of *AOX1* mRNA. The expression level of HSA is increased >50 fold by optimizing the 5′UTR to mimic that of *AOX1* mRNA (18). Expression plasmids such as pHIL-D7 (42) can be used to make an exact construct. This plasmid has unique *Asu* II and *Eco* RI sites immediately following 5′*AOX1*. The second *Asu* II site that was originally present in 3′*AOX1* has been eliminated. Therefore, the sequence TTCGAAACG can be added immediately upstream of the ATG start codon of the gene of interest, and an *Eco* R I site can be engineered downstream of the stop codon for insertion at *Asu* II–*Eco* RI sites of pHIL-D7 (42).

Transcriptional Optimization

Genes with high A + T nucleotide clusters are poorly transcribed in *Pichia* due to premature termination of the transcription. For example, ATTATTTTATAAA, present in HIV-gp120 has been identified to block transcription in *P. pastoris*, and the premature termination is overcome by altering the sequence to TTTCTTCTACAAG (22). Because we are not aware of all the problematic A + T rich clusters, a general strategy with A + T rich genes is to redesign them using *P. pastoris* preferred codons (48,54), http://www.kazusa.or.jp/codon/) so as to have an A + T content in the range of 30–55%. By using this approach, it has been possible to construct *Pichia* strains for efficient production of several proteins, which include tetanus toxin

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fragment C (20), *Bacillus sphaericus* BSP1 and BSP2 heterodimeric mosquitocidal toxin (55), anticoagulant protein ghilanten (56), spider silk proteins (57), human endostatin (58), and human lactoferrin (59).

Product Secretion

For a protein which is normally secreted, that is the preferred mode of expression, for multiple reasons. It is easier to recover product from extracellular medium as there will be relatively fewer proteins. Also, many proteins that are normally secreted remain predominantly insoluble when expressed in the intracellular compartment, as has been seen with HSA and salmon growth hormone (unpublished observations). Likewise, a protein that is not normally secreted may be difficult to secrete. However, in some cases, such as human TNF, it is possible to express at high levels in the intracellular compartment (16). Interestingly, the intracellular human tetrameric catalase has successfully been expressed as a secreted protein in *Pichia* (60).

A wide variety of heterologous proteins have been secreted in *Pichia*. In several instances, HSA (18), invertase (17), bovine lysozyme (61), barley alpha amylases (62), cathepsin E (63), and thaumatin (64) the native signal sequence is adequate. In the case of matrix metalloproteinases, although native signal sequences work (41), both secretion and product yield are improved while using the *S. cerevisiae* pre-proalpha mating factor (αMF) secretion signal sequence (65,66). Likewise, with *Candida rugosa* lipase 1, although native signal works, both product stability and yield are tremendously improved with the use of pre-proαMF signal sequence (67). For thaumatin secretion, native signal works, but not the pre-proαMF signal sequence (64), whereas for human interferon-alpha 2b (IFN-a2b), native signal sequence does not work, but pre-proαMF works (68).

Pre-proαMF secretion signal sequence has worked well for secretion of large variety of proteins, including the smaller-sized products such as aprotinin (69), EGF (19,69), IGF-1 (70), and ghilanten (56). The processing of pre-proαMF secretion signal involves three steps. The first is the elimination of the pre-region by signal peptidase, second, KEX2, and YPS proteinases cleave out the proregion (71,72), and finally, N-terminal Glu–Ala repeats are removed by the action of dipeptidyl-aminopeptidase (Dpap). The efficiency of each processing step depends on amino acid sequence adjacent to the processing site as well as the tertiary structure of the secreted protein. All these factors contribute to incomplete processing and/or reduced yield of mature protein (73–75).

In making αMF signal sequence constructs, it is generally preferable to retain the Glu-Ala spacers adjacent to the Kex2-like protease cleavage site (... Val-Ser-Ser-Leu-Glu-Lys-Arg-$^{\text{Kex2p}}$Glu-Ala-^{Dpap}-Glu-Ala-^{Dpap}-fused protein). The presence of Glu-Ala spacers help to alleviate the steric interference imposed by the fused protein, resulting in an efficient cleavage of the prosequence by the *Pichia* Kex2 like protease (69). The Glu-Ala spacer is subsequently cleaved by diamino peptidase (Dpap coded by *STE13*) to yield the protein of interest free of additional N-terminal amino acid residues. Interestingly, it has recently been reported that the pre-proαMF without the Glu-Ala repeats directed the secretion of correctly processed human IFN-a2b at 200 mg/L level, whereas the pre-proαMF having the Glu–Ala spacer, although secreted an equivalent amount of IFN-a2b, the secretion signal was inefficiently processed (68). Thus, ideally all variations must be explored to arrive at the best fit for a given protein.

Other secretion signal sequences used in *Pichia* include those of acid phosphatase (Pho1p), hybrid of Pho1p secretion signal with αMF and Kex2p cleavage site, invertase, lysozyme, HSA, *Phaseolus vulgaris* agglutinin, inulinase, and alpha amylase, glucoamylase, and *S. cerevisiae* killer toxin I (42,59,76).

Choice of the Secretion Signal

Only in a limited number of cases has a thorough comparison been made on the relative efficacies of different signal sequences and variations thereof (for example, (59,64,68,69)). In the case of invertase secretion, both the extent of glycosylation and secretion rate are enhanced when the native signal sequence is substituted with the pre-proαMF signal sequence (69), although both yields are greater than gram per liter level of the product. However, in the case of proteins that are more susceptible to proteolysis, improvement in secretion rate can increase production level as has been noticed with MMP-1 (41,65,66). Based on the relatively high success of pre-proαMF signal sequence, it makes sense to test that signal (and variations there of) first, side by side with the native secretion signal sequence, before exploring other signal sequences.

Production Enhancement by Manipulation of Media and Growth Conditions

More often than not, a secreted protein is rapidly degraded in the *Pichia* medium due to extracellular proteases, cell-bound proteases, as well as by intracellular proteases released to medium due to cell death and cell lysis. Multiple stress factors (especially in high cell density fermentor), which include starvation, pH shift, temperature change, change of carbon source, buildup of toxins, and reactive oxygen species (ROS) are considered to cause cell death and cell lysis (77).

One of the approaches to increase the stability and yield of the secreted protein in the culture medium is by manipulating the pH of the medium to arrive at the optimal pH for blocking a problem protease. Suggested pH range for experimentation is between 2.8 and 8 (see section titled "Media Compositions"). This pH range does not affect the growth significantly. HSA yield was significantly improved by raising the pH from 5.2 to 6, with adequate aeration. The yield was further enhanced by the addition of yeast extract (1%) and peptone (2%) (45,48,78,79). Production of mouse EGF was favored at pH 6 in the presence of casamino acids (19). Casamino acids is preferable to yeast extract + peptone, because the peptide components of peptone (such as collagen fragments) can interfere in product analysis and recovery (41). For both IGF-I (70) and cytokine growth-blocking peptide (80), pH 3 was found to be optimal. Greater than twofold increase

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in production of cellulose-binding module cellulose 6A and lipase B (CBM-CALB) is noted by reducing the pH from 5 to 4 in a bioreactor run (81).

Hirudin variant 2 (rHV2) secreted in *Pichia* was found to be degraded to four active fractions, including the intact rHV2 (Hir 65) and three C-terminally truncated forms Hir 64, Hir 63, and Hir 62 (82). Degradation of hirudin was reduced by using growth-rate-limiting quantities of methanol (3.09 g/L methanol; specific growth rate maintained at 0.02–0.047 h^{-1}) (82). Degradation of hirudin was also reduced by increasing NH_4^+ to relieve the stress of nitrogen starvation (83). Ascorbic acid (4 mmol/L) was used to quell ROS and improve cell viability. Under these conditions intact and total hirudin production reached 2.90 and 5.03 g/L, respectively, in contrast to 1.75 and 4.70 g/L in the absence of ascorbic acid (84).

Lower cultivation temperature is shown to improve product yield in several instances, which include CBM-CABL (22°C) (81), laccase (20°C) (85), galactose oxidase (25°C) (86), Rh midkine (20°C) (87), human AFP (23°C) (88), herring proteins (23°C) (89), and human bile salt activated lipase (BSSL, 20°C) (90). *Pichia* cultivated at temperatures as low as 15°C, greatly enhanced the yield of ScFV production due to reduced protease level (91). Secretion level of bivalent T cell immunotoxin A-dmDT390-bisFy(G_4S) was also enhanced at 15°C, which was attributed to reduced protease levels, improved folding, as well as reduced toxicity of the immunotoxin to *Pichia* (92). Thus, we may say that the low temperature expression helps to increase the yield of aggregation-prone, unstable, and toxic products in *Pichia*.

Addition of 5 m*M* EDTA to the medium also improves accumulation of proteins expressed in *Pichia* (41). Supplementation of the induction medium with 0.4 *M* L-arginine, 5 m*M* EDTA, or 2% casamino acids in the BMMY induction medium (see section titled "Media Compositions") increased scFV expression (91). It should be noted that medium manipulation can significantly alter the profile of endogenous protein components in the culture media, such that previously unnoticed proteins accumulate at high levels. For example, we have noticed that addition of 5 m*M* EDTA causes accumulation of a protein of approximately 50 kDa in the extracellular medium (41). This protein has the aa sequence DIIWDYSSEKIMGVNLGGWL..., which matches closely with the exo-β-1,3-glucanase of *S. cerevisiae* (93) and *Candida albicans* (94). We have also noticed that overexpression of human tissue inhibitor of matrix metalloproteinase (TIMP1) in *Pichia* leads to greater gram per liter accumulation of a 18–22 kDa *Pichia* protein of sequence ADYMC?GLAIYGAWEC?GPEAGPFDSEC?LLATD (41).

Production Enhancement Using Protease-Deficient Strains

In addition to the optimization of media and growth conditions, the product yield can be further improved by using a protease-deficient *Pichia* strains generated by knocking out a *Pichia* protease or by overexpressing a protease inhibitor (for example, TIMP1 strain as noted above).

Protease-deficient *Pichia* strains SMD1168 (*his4*, *pep4*), SMD1165 (*his4*, *prb1*), and SMD1163 (*his4*, *pep4*, *prb1*) have been constructed (95). These strains have a disruption in the genes encoding proteinase A (*PEP4*) and/or proteinase B (*PRB1*) (56). Proteinase A is a vacuolar aspartyl protease necessary for the activation of vacuolar proteases, such as carboxy peptidase Y and proteinase B. In the absence of proteinase A, *Prb1p* is not fully active. Thus, *pep4* strain (SMD1168) is also expected to have a lower level of Proteinase B activity. The *prb1* strain (SMD1165) lacks proteinase B, whereas *pep4prb1* strain (SMD1163) lacks all three of the protease activities. These strains in general have low storage viability and are less robust (42,43). Nevertheless, these protease-deficient strains, in combination with other strategies to improve product stability, have been used to improve the production yield of IGF-1 (46), ghilanten (56), laccase (96), and human catalase (60). Disruption of *KEX1*, which codes for a carboxy peptidase, resulted in expression of full-length endostatin in *Pichia* (97). Recently, using a gene pop-in/pop-out gene replacement approach (98), the *KEX1* was deleted from a *Pichia* hirudin production strain. This resulted in most significant improvement in intact Hir65 production, which approached 2.4 g/L for the *KEX1* deleted strain compared to 1.1 g/L seen with the strain without *KEX1* deletion (99).

Enhancement of Protein Secretion by Overexpression of Chaperone Proteins

Pichia is able to perform many posttranslational modifications found in higher eukaryotes, which include correct folding, disulfide bond formation. Folding and disulfide bond formation in some cases can be the rate-limiting step in protein expression leading to protein aggregation (100). It is not clear which single chaperone is most important or which combination optimally cooperates in this process.

Overexpression of *Pichia* protein disulfide isomerase (PDI), which is important for protein folding in the endoplasmic reticulum (ER), was able to increase the secretion of Na-ASP1 protein in high copy clones (47). As noted before, high copy clones of Na-ASP1 secreted less material than single-copy constructs, perhaps due to overburdening the *Pichia* secretory/protein folding machinery, which was corrected by PDI overexpression.

A33scFV in *Pichia* is expressed at 4 g/L level, which rose to >10 g/L by overexpression of immunoglobulin binding protein (Bip) (101). The noted impressive increase is attributed to increase in folding capacity. PID overexpression did not have any effect on A33scFV expression. This was unexpected because A33scFV contains disulfide bonds. Furthermore, simultaneous overexpression of both BiP and PDF also did not have any effect in this system. It was also noted that PDI expression in the A33scFV strain caused a six-fold increase in endogenous BiP expression, suggesting that PDI was inducing an unfolded protein response due to excess chaperone and recombinant protein in the ER. In another study it was found that the chaperone combinations YDJiP/PDI, YDJiP/Sec63, and Kar2p/PDI synergistically increase secretion levels 8.7, 7.6, and 6.5 times, respectively (102).

A transcriptomics-based approach to identify novel factors enhancing heterologous protein secretion by analyzing

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differential transcriptome of a *Pichia* strain overexpressing human trypsinogen versus that of a control strain has identified 524 genes to be significantly impacted. This was narrowed down to 13 genes, all upregulated in the expression strain, as potentially important. The respective *S. cerevisiae* homologs of these genes were cloned and tested. All genes except one showed a positive effect on Fab fragment secretion, of which *PDI1*, *ERO1*, *SSO2*, *KAR2*, *BiP*, *and HAC1* are previously characterized secretion helpers; the newly identified ones to have role in product secretion are *Bfr2* and *Bmh2* (involved in protein transport); *Ssa4* and *SSe1* (chaperones); *Cup5* (vacuolar ATPase subunit); and *Kin2* (a protein kinase subjected to exocytosis) (103). The chaperone game has just begun and we can foresee exploitation of this approach more fully in the years to come.

Protein Glycosylation and "Humanization" of *Pichia* N-linked Glycosylation

Pichia is able to perform both O-linked and N-linked glycosylation of secreted proteins (17). O-linked glycosylation involves addition of oligosaccharides to the hydroxyl groups of Serine and Threonine residues of the secreted proteins. The O-linked residues in mammalian cells are composed of variety of sugars (*N*-acetyl glucosamine, galactose, sialic acid), whereas in *Pichia* it is solely made up of mannose sugar. There is no defined consensus primary amino acid sequence for O-glycosylation and different hosts may O-glycosylate at different positions of the same protein. Even if a protein is not O-glycosylated in the native host, it may receive O-glycosylation in *Pichia*. For example, IGF-1 is not O-glycosylated in human host, whereas in *Pichia*, about 15% of the IGF-1 produced is O-glycosylated (46).

N-linked glycosylation involves addition of oligosaccharide chains to Aspargine at the recognition sequence Asn-X-Ser/Thr. It begins in the ER with the transfer of a lipid-linked oligosaccharide unit, Glc(3)Man(9)GlcNAc(2) (Glc = glucose; Man = Mannose; GlcNAc = *N*-acetyl glucosamine) to Asn of Asn-X-Ser/Thr (104). This unit is then trimmed to Man(8)GlcNAc(2). At this point onwards, the lower eukaryotes and higher eukaryotes deviate in the subsequent modifications that take place in the Golgi apparatus.

The mammalian Golgi performs a series of trimming and additions leading to three classes of N-linked glycans: "high mannose" [Man(5–6)GlcNAc(2)], "complex" (mixture of several different sugars) and "hybrid" (combination of the high mannose and the complex) with a common core, Man(3)GlcNAc(2). In *Pichia* secreted glycosylated proteins (native as well as heterologous) only "high mannose" kind typically with 8 to 9 mannose residues in length [Man(8–9)GlcNAc(2)] predominate with smaller amounts of chains with more mannose residues. In *S. cerevisiae*, only "hypermannose" kind with 50–150 mannose residues in length [Man(50–100)GlcNAc(2)] are present resulting in a hyperglycosylated protein. *Pichia* appears not to have any terminal α-1,3 linked mannose (105,106) that are unique to fungal glycoproteins and render recombinant proteins produced in fungi unsuitable for human pharmaceutical uses (105). This should not be misconstrued to think that *Pichia*-produced glycoproteins are perfect for human pharmaceutical use, because with only "high mannose" kind, it is a far cry from mammalian-type glycosylation. The high mannose glycans are believed to reduce the half-life of the glycoprotein *in vivo* and may be immunogenic, thus limiting the potential therapeutic value of *Pichia* derived glycoproteins (107). Over the past two decades, several groups have attempted to overcome this problem, yet with limited success. Recently, however, major advances in the glyco-engineering of the *Pichia* yeast have culminated in the production of fully humanized sialylated glycoproteins (108). With the advent of glycoengineered *Pichia* host with humanized N-linked glycosylation potential, the goal of production of authentic human glycoproteins in *Pichia* is getting closer than ever (59,109).

Coexpression of Proteins and Proteins Complexes

Coexpression approach may not only be useful for increasing expression, often it may be necessary to express two or more heterologous proteins at the same time. The first example of coexpression is that of the BSP1 (43 kDa) and BSP2 (52 kDa) components of *B. sphaericus* mosquitocidal toxin (55). Since then, there have been other reports of coexpression (47,101–103,110,111). Many proteins function as members of multicomponent complexes and successful expression strategy necessitates coexpression of subunits (39–41,110). The simplest way to engineer strains for coexpression is by sequential transformation or by cotransformation. Numerous dominant selection markers are available, which can be readily used for introducing multiple constructs at the same time.

Fusion Proteins

Fusion protein expression has many advantages. Often it can help stabilize a protein, and can also be valuable for expression of peptides. The most popular fusion partner is the highly expressed HSA (112). Often therapeutic proteins fused to HSA can be used directly for therapeutic applications. Albuferon is a long-lasting fusion protein between human interferon α-2b and HSA, which is being codeveloped by Human Genome Sciences and Novartis for treatment of Hepatitis C (113). Both fusion configurations HSA–IFN α-2b and IFN α-2b–HSA were expressed in *Pichia*. Although both expressed well, and showed comparable antiviral activity, IFN α-2b–HSA was more homogenous and stable. Heterogeneity and instability of HSA–IFN α-2b was caused by incomplete disulfide bridge formation between Cys^1 and Cys^{98} of IFNα-2b due to structural perturbation of IFN α-2b by HSA. Other active fusion proteins successfully expressed in *Pichia* include HSA–PTH (1–34,114).

Overcoming Protein Toxicity

Occasionally the protein being expressed may be toxic to *Pichia*. Whether a protein is toxic to *Pichia* can be ascertained by comparing the growth of transformants on 1% sorbitol (or 100 m*M* alanine) as sole carbon source to

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that on sorbitol or alanine + methanol. Because, sorbitol and alanine are nonrepressive carbon sources (42,115), methanol is able to induce *AOX1* promoter in their presence. The other nonrepressive carbon sources include mannitol and trehalose (115). If the expressed protein is toxic, the growth in the presence of methanol will be drastically impaired. Even if the protein is toxic, it is still possible to express, because of the tight regulation of *AOX1* promoter. A stepwise induction is suggested. First the cell mass is built up using glucose as sole carbon source, taking care to see that glucose is not limiting. Under these conditions *AOX1* promoter remains completely repressed. Subsequent to this, protein production is initiated with methanol feed.

Alternative Promoters for Expression

Although *AOX1* promoter has been used extensively, with great success, several other promoters are available for exploration. The only potential drawback with the *AOX1* promoter, if we may say so is the need for methanol, which is a potential fire hazard especially in quantities needed for megaton plants. Also, the use of methanol may not be appropriate for production of food products. Furthermore, the unique, unsurpassed tight regulatory and highly inducible features of *AOX1* promoter may not be needed in every instance. Thus, promoters that are not induced by methanol may be useful in some instances for expression of certain genes. The following are alternative promoters:

1. The constitutively active *GAP* (glyceraldehyde-3-phosphate dehydrogenase) promoter (116).) The advantages of this promoter is that the protein can be produced more straightforwardly without having to make media shifts and the promoter strength is quite high. It is also convenient for production of isotope labeled proteins for NMR studies. GAP promoter is not a good choice for the production of proteins that may be toxic.
2. Methylamine (or methanol) inducible *FLD1* (formaldehyde dehydrogenase) promoter (117). This retains many of the tight regulatory features of *AOX1* promoter. Promoter strength is quite high. A key advantage is that it can be induced using methylamine, which is inexpensive, as a sole nitrogen source (with glucose as carbon source). It is repressed in medium with glucose and ammonium sulfate.
3. Methanol or oleate inducible *PEX8* (a peroxisomal matrix protein gene) promoter (118). The promoter strength on methanol is considerably weaker than that of *AOX1* or *FLD1* promoters.
4. Constitutively active weak *YPT1* (a GTPase involved in secretion) promoter (119). The YPT1 promoter provides a low but constitutive level of expression in media containing glucose, methanol, or mannitol as carbon sources (119). This may be useful for coexpression of chaperones to enhance protein folding and secretion in production strains.
5. Ethanol and stationary phase inducible ICL1 (isocitrate lyase 1) promoter (120). ICL1 promoter is inducible with ethanol and repressed by glucose in the exponential phase. In the stationary phase ICL1 is expressed even in the presence of glucose.

One aspect that investigators have to keep in mind in using these alternate promoters for commercial applications is that they may have to pay royalty as the patents are still active, unlike *AOX1* promoter patent, which has expired.

FERMENTATION PROCESS

Typically two-phase fermentation is used. In the first phase (biomass stage), cells are grown until glycerol is depleted. In the second phase (protein production stage), gene expression begins by feeding methanol to the bioreactor. There are numerous factors that impact production yield in a bioreactor, including culture medium composition, strain, pH, temperature, agitation rate, dissolved oxygen, induction condition, and fermentation strategy. Several of these factors have been already discussed (see section titled "Production Enhancement by Manipulation of Media and Growth Conditions". Basic protocols for *P. pastoris* fermentation have been abundantly described in literature (e.g. (42,121), and http://www.biotechlab.net/UserFiles/File/787348_Ref_Invitrogen2002.pdf.). Improvements which have been made to basic protocol have been recently reviewed (122,123). Few salient features are reviewed here.

Fermentation Media and Operational Conditions

Basal salt medium (BSM) is the most common medium for high cell density fermentation, although it may not be the optimum and may have some important problems (unbalanced composition, precipitates, ionic strength, etc.). Therefore, alternative media such as FM21 (44) and FM22 (121,122) have been described. The BSM medium provides, in general, high concentration of basic elements, all at the upper level of the recommended range proposed by Wegner (3). Conversely, FM21, and the D'Anjou media (123) have a low concentration of chemical elements, around the low limit suggested by Wegner (3). The concentrations of the elements in FM22 media are similar to BSM with the exception of potassium. One of the most important points in a medium formulation is the nitrogen source. In BSM, and FM21, nitrogen is solely added as ammonium hydroxide when controlling pH. This is also true to a large extent for FM22. However, in the D'Anjou medium all nitrogen is supplied at the initial formulation and it is not added during the culture. Nitrogen starvation starts around 50 g/L of biomass (123). The lack of nitrogen is directly related to the increase in proteolytic activity resulting in the degradation of extracellular protein. Thus, in BSM and FM22 media, where nitrogen is added to control pH, this effect could be present when high biomass concentration is reached. The optimal conditions (medium, pH, temperature, etc.) are dependent on the strain used and thus have to be adjusted accordingly

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using the guidelines and considerations noted (see section titled "Production Enhancement by Manipulation of Media and Growth Conditions").

Dissolved Oxygen (DO) Control

Dissolved oxygen (DO) is an important factor for protein productivity in a bioreactor. It can be controlled with combination of agitation rate, and the air (or O_2) flow rate. During the glycerol batch phase (20–24 h), the DO is controlled by adjusting the agitation rate up to 800 rpm for a typical 4-L bioreactor, and around 450 rpm for 60-L reactor, and then manually adjusting the air (or O_2) flow rate into bioreactor. Upon exhaustion of glycerol, the DO will rise rapidly. Typically, DO should be maintained around 30%, but need to be further optimized on a case by case basis.

Growth Kinetics

In the methanol unlimited fed-batch and continuous cultures with Mut^+ cells, growth inhibition at high methanol concentration is noted. Specific growth rate increases with methanol concentration up to a maximum value named theoretical maximum specific growth rate μ'_{max}, corresponding to a critical substrate concentration (Scrit), beyond which it declines. Methanol concentration lower than Scrit, represent growth-limited state and for values higher than Scrit, represent the growth inhibited state. A comparison of the kinetic parameters obtained for the different heterologous proteins indicate that the growth rate is influenced by the protein being expressed (123).

Fed-Batch Fermentation Operational Strategy

Fed-batch fermentation protocols include three different phases. A glycerol batch phase (GBP), a transition phase (TP), and finally a methanol induction phase (MIP).

1. *Glycerol Batch Phase (GBP):* The objective of GBP is to rapidly generate biomass prior to protein production. The maximum specific growth rate of wild-type *Pichia* on glycerol (0.18 h^{-1}) is higher than growing on methanol (0.14 h^{-1}) (69) and this methanol μ_{max} is, generally, lower when *Pichia* is producing a heterologous protein. GBP strategy is common for Mut^+, Mut^-, and Mut^S *Pichia* strains and typically 40 g/L glycerol is used; concentration over 40 g/L could inhibit growth. Brierley and coworkers recommended a maximum glycerol concentration of 6% (70) and ethanol levels between 0.5 and 2.4% were detected when glycerol concentration was over 7% (124). The observed biomass to substrate yield (YX/S) (biomass expressed as dry cell weight) is about 0.5. Thus, a final biomass around 20 g/L is obtained at the end of GBP. The glycerol exhaustion (end of batch phase) is indicated by a sharp increase in the DO, which is the most common parameter used for initiation of TP.
2. *Transition Phase (TP):* TP has two objectives, one is to further increase biomass level and the other is to cause de-repression of the *AOX1* promoter due to the absence of excess glycerol prior to MIP. Constant glycerol feeding rate (125) or an exponential glycerol feeding rate is used to reach a growth-limited level. Cell growth predictions, for different exponential feeding profiles have been shown by Zhang and coworkers (126). The maximum glycerol specific consumption rate is 0.0688 g/(g h). The final biomass levels reached at the end of the TP is generally above 30 g/L. During TP, glycerol feeding rate is generally complemented with a methanol feeding rate to augment the de-repression of *AOX1* promoter. With mixed feeding, cultures can be primed for methanol induction, which leads to a substantial reduction in the length of MIP, as has been demonstrated in lipase production (127). Also, the methanol supplement can strongly support cells to synthesize *AOX1* (128). Different methanol profiles have been used for the TP, which include the following:
   - raising methanol feeding rate as a function of response of DO up to a 7.6 mL/(h L) (129);
   - maintaining methanol concentration at set points 4 g/L (125) or 1–2 g/L (130);
   - about 1 h starvation phase prior to MIP to assure that glycerol is completely consumed (131);
   - a decreased glycerol feeding rate and constant methanol feeding rate (132,133).
3. *Methanol Induction Phase (MIP):* The methanol feeding strategy, which also dictates the specific growth rate, is one of the key factors for maximizing protein production (126,128), since protein production is directly or indirectly associated with cell growth (134). Moreover, MIP may depend on the operational conditions (e.g. temperature, pH, and culture medium), phenotype, and specific characteristics of the heterologous protein produced. The synthesis, processing, and secretion of the protein could also affect cell growth. Different approaches have been proposed to optimize MIP. These strategies are reviewed in (123).

Methods for Monitoring Methanol Concentration

One of the key parameters of *AOX* promoter based *Pichia* expression system is the methanol concentration, which is both a substrate and inducer. High levels of methanol are toxic to the cells and low levels of methanol may not be adequate for induction of expression. For optimal protein production, methanol induction strategy is critical. This has to be implemented keeping in mind the genetic background of the strain (Mut^+, Mut^S, or Mut^-), which has been addressed in the fermentation guidelines provided by Invitrogen Corporation (http://www.biotechlab.net/UserFiles/File/787348_Ref_Invitrogen2002.pdf) and in other published reviews (42,121–123). Keeping a constant methanol concentration during the induction phase is beneficial to optimal protein production (121). DO spike method has been used historically to estimate the methanol concentration. This technique, however, exposes the cells to potentially noninducing levels of methanol and cannot be easily implemented with Mut^- clones or with mixed substrate feeds. Off-line methods for methanol

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quantification include gas chromatography, HPLC (high performance liquid chromatography), and enzymatic determination, which are used in connection with a batch or fixed preprogrammed methanol addition strategy. A near infrared spectroscopy (NIRS) technique has also been used to model key analytes (biomass, glycerol, methanol, and product concentration) in the production of a therapeutic protein in high cell density culture (135). These off-line methods require a pretreatment of the sample and there will be precious time lapse for implementation. The sequential injection analysis (SIA) developed by Surribas and coworkers is able to measure seven samples per hour with low standard deviation (136). YSI 2700 select analyzer (http://www.ysilifesciences.com/) from Yellow Springs Instruments (Yellow Springs, Ohio) can be readily connected to a bioreactor for SIA. However, continuous extraction of samples increases contamination probability, besides sample loss.

Fortunately, many on-line methanol sensors have been successfully adopted to monitor *Pichia* fermentors (123), which are as follows:

- Raven methanol sensor from Raven Biotech Inc. (Vancouver, BC, Canada), which uses a probe inserted into the bioreactor (http://www.raven-biotech.com/products.html).
- TGS822 sensor for the detection of organic vapors from Fiagro Electronic Co. Ltd (Tahin, China) (http://www.etesters.com/products/details.cfm/part/TGS822/productID/58856492-802e-f41e-d604-2d325dd53216).
- Directly detecting the methanol vapor in the stream gas outlet by diverting it to an MC-168 methanol monitor and controller (PTI Instruments, Inc., Kathleen, Georgia) equipped with a TGS822 methanol sensor.
- Alkosense probe (Frings America, Illinois) (137). This measuring principle is based upon the selectivity of the semipermeable membrane system, which rejects low volatility substances like sugar and glycerin, as well as most organic substances including organic acids. If easily volatilized components are present, that is ethanol and methanol, the system will not be able to differentiate between the various components, and will measure the combined concentrations. (http://www.fringsamerica.com/markets/brewing/alkosens.asp).

If the growth kinetics of a *Pichia* production strain is preestablished, then the methanol feed rate can be adjusted according to the specific growth rate during the induction phase. This can be an effective way to maintain optimal methanol concentration in the bioreactor. In fact, this strategy has resulted in 2.5-fold higher productivity of lipase (127). To improve the expression level, glycerol and methanol were fed simultaneously, followed by a single methanol feed, and resulted in the highest productivity [12,888 U/(L h)], which is 13.6-fold higher than the DO-based strategy. On-line methanol measurement, which permits implementation of closed-loop control algorithm (138) can be more effective than open-loop control strategies or indirect methanol feed rate from other fermentation parameters/variables as specific growth rate in particular, from industrial point of view for a reproducible fermentation at a constant methanol set point.

Methanol Control Schemes

To produce small quantities of heterologous protein, the methanol feeding strategy from http://www.biotechlab.net/UserFiles/File/787348_Ref_Invitrogen2002.pdf or as found in (42) is adequate. When the objective is to reproducibly maximize product yield and for quality control, the selection of the optimal methanol feeding strategy in the induction phase is necessary. When on-line methanol concentration monitoring is not available two different methanol feeding strategies, DO spike method, and an open-loop methanol control strategy are most commonly used (121). Both these strategies and their limitations have been elegantly reviewed (123). In the DO spike method, methanol feeding rate is modified in order to avoid methanol exhaustion as indicated by a sharp increase in dissolved oxygen. Neither methanol concentration nor specific growth rate are constant in this strategy, and thus it is difficult to study the effect of these variables on the production of the heterologous protein. DO spike method has an additional problem, if an inhibitory methanol level is reached, a sharp increase in DO will be observed, the response of the system will be then to increase the methanol feeding rate leading to methanol accumulation to toxic levels in the bioreactor. In the method based on the methanol control using an open-loop structure, a feeding rate profile derived from mass balance equations to theoretically maintain a constant specific growth rate (μ) is used. This approach is based on simple cell growth models with no on-line information about the system. To implement this method, a constant YX/S for the fed-batch induction phase is considered and, biomass concentration and volume are supposed to be known at the beginning of the preprogrammed feeding strategy. Zhang and coworkers, empirically developed a methanol feeding strategy based on a growth model, obtaining μ to maximize the protein production (126). D'Anjou and Daugulis have demonstrated the usefulness of a rational, model-based approach for improving the productivity by using an exponential feeding strategy with mixed glycerol–methanol substrate in a fed-batch (130). Although open-loop systems could be easy to implement they do not respond to possible perturbations of the system. Simplest closed-loop control strategy is the "on–off" control mode. Some works have been published with apparently satisfactory results (for example, (126,133)). However, due to the complexity of the system, a simple "on–off" control strategy is inadequate for precise control of methanol concentration in the bioreactor leading to fluctuations in methanol concentration around the set point.

Mixed Substrates

One strategy to increase the productivity of *Pichia* expression is the use of a multicarbon substrate in addition to methanol. It is a simple way to augment the energy supply to cells in the culture broth (128,133,139). A mixed feed

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reduces the induction time, increases cell density, and volumetric productivity (140). The mixed feeding strategy is generally employed for Mut^s *Pichia* fermentations because of the slow methanol utilization, which requires large induction times (above 100 h), although it has also been used for Mut^+ strains (141). Glycerol has been the most feeding cosubstrate used at limited rates, ensuring good cell growth while inducing the expression of the heterologous protein. However, although the volumetric productivity increased, the specific productivity of recombinant protein is lower because glycerol represses the *AOX1* promoter (142). Inan and Meagher compared different carbon sources in terms of their ability to support growth and expression of an *AOX1-lacZ* fusion in shake flasks studies of a *P. pastoris* Mut^- strain (115). As predicted, glucose, glycerol, ethanol, and acetate supported growth but repressed the expression of β-gal. On the contrary, growing on media containing alanine, mannitol, sorbitol, and trehalose expressed as much or higher amount of β-galactosidase compared to the Mut^+ strain. Recently, lactic acid has also been referred as nonrepressing substrate (142). The use of a less repressing carbon source may result in higher specific production rates, improving overall productivity, and eliminating the need for such tight control of residual substrate levels. Among them, sorbitol is a widely accepted nonrepressive carbon source for *P. pastoris* and methanol can be added to the sorbitol feed at any desired point to initiate induction of expression (41).

Continuous Fermentation

Continuous production mode in comparison to fed-batch fermentation, offers advantages in terms of higher volumetric productivity, product quality, product uniformity, significant reduction in exposure of the product to the proteolytic enzymes, oxidative damage or inactivation, and overall reduction of downtime (143,144). Continuous operation using methanol as sole carbon source under control of *AOX1* promoter is practically limited to Mut^+ cells. The low maximum specific growth rate of Mut^s phenotype limits the operational dilution rate and thus, the productivity of the process. However, Boze and coworkers in a continuous process at a dilution rate of 0.012/h with a Mut^s obtained a 6.4-fold higher productivity and 2.3 times higher specific productivity of porcine Follicle-stimulating hormone (FSH) compared to that achieved with fed-batch process (145). As discussed under "mixed substrate" section, one approach to increase the productivity of Mut^s phenotype is the use of a mixed carbon feed. Glycerol and sorbitol are the most commonly used cosubstrates jointly with methanol. With glycerol, the continuous strategy is to select a dilution rate far enough from μmax to ensure that no glycerol is allowed to accumulate in the broth, and also methanol concentrations have to be maintained at levels sufficient to fully induce heterologous protein production, yet not so high as to be inhibitory to cell growth or heterologous protein expression (130). D'Anjou and Daugulis performed a set of continuous stirred tank reactor or CSTR (continuous fermentation) experiments to determine the relationship between the dilution rate (in a range between 0.01 h^{-1} and 0.09 h^{-1}), the specific methanol consumption rate, and the specific production rate in the heterologous production of a sea raven antifreeze protein using mixed methanol–glycerol feed (146). The specific methanol consumption rate as a function of the dilution rate showed a maximum at approximately $D = 0.05\ h^{-1}$. Maximum production was achieved at the lowest dilution rate. However, the maximum specific production rate (Q_p) was achieved at the highest dilution rate. This behavior confirms that product formation is related to growth by a constant yield coefficient. Finally, they demonstrated that a CSTR system will yield a higher productivity compared with a fed-batch system. Readers are directed to Refs (122) and (123) for further discussions on CSTR.

Cyclic Fed-Batch Culture (CFBC)

A simple cyclic fed-batch culture (CFBC) consisting of a constant medium feed with periodic withdrawals of culture resulted in a product yield (13.4 mg HSA/g biomass) similar to that obtained using the complex multiphase industrial production strategy (13.7 mg HSA/g biomass) has been reported (147). Thus, using a much simpler protocol, it is possible to obtain yields comparable to that seen with more complex multiphase feed strategy.

Hypoxic Fed-Batch Cultivation

High cell density fermentation of *Pichia* has to cope with many technical limitations, most importantly the transfer of oxygen. By applying hypoxic conditions to chemostat cultivations of *Pichia* expressing an antibody Fab fragment under the GAP promoter, a 2.5-fold increase of the specific productivity Q_p at low oxygen supply was observed. At the same the biomass decreased and ethanol was produced, indicating a shift from oxidative to oxidofermentative condition. Based on these results a feedback control for enhanced productivity in fed-batch processes, where the concentration of ethanol was kept in the culture at 1% (v/v) by regulated addition of feed medium. This strategy was tested successfully with three different production strains, leading to a three- to sixfold increase in Q_p and threefold reduced fed-batch times. Taken together, volumetric productivity Q_p increased 2.3-fold (148).

Optimization of Trace Metals

A study of trace metals effect on growth and yield of LACZ production in GS115 Mut^+ strain has shown that both Mg and Zn are essential to support growth, but the amount required is considerably lower than the level typically used. Supplementation with Ca, Co, Mo, Fe, Mn, I, and B were not required to sustain cell mass. When the medium was reformulated with only Mg and Zn, the cells grew for 12–15 generations, which are expected for high cell density fed-batch fermentation. Product yields of LACZ was significantly affected by trace metals. When optimizing a fermentation process, the objective is also to decrease production costs, including indirect issues such as compliance to regulatory agency restrictions, impacts to downstream processing steps, effect of the medium components to equipment maintenance, etc. Clearly, reduction

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or elimination of trace metals potentially impacts all these intangibles (149). This study shows the importance of critically examining and accordingly adjusting the trace metal levels for a given production strain.

CONCLUSIONS AND FUTURE PERSPECTIVES

Pichia undoubtedly has proven to be a reliable system for expression of a wide assortment of proteins, many of which are expressed at several grams per liter levels. It is impressive to see how enthusiastic researchers from all corners of the world are willing to make it work, because it seems as though a researcher feels, it is his/her own fault, if the protein of interest did not work in *Pichia*! This bears testimony to the grand track record of *Pichia*. With the recent success seen in expression of several membrane proteins, I am sure we will hear many more success stories in that category as well. With the availability of glycoengineered *Pichia*, we can foresee that *Pichia* will gradually replace mammalian cell expression system for production of authentic human pharmaceuticals. *Pichia* surface display has just surfaced (150); we can anticipate all sorts of novel surface display applications in the years to come. *Pichia* expressing fish growth hormone has been directly fed to fish to dramatically impact growth rate (151); *Pichia* expressing *B. sphaericus* mosquitocidal toxins (55) is an effective biopesticide when fed to mosquito larvae, and likewise, *Pichia* expressing garlic lectins (152) shows potent insecticidal activity toward aphids. With wider acceptance of genetically modified organisms, we can foresee wholesome *Pichia* applications in biopesticide (55,152), biocatalysis (153,154), and perhaps even *Pichia* burger—why not? It is able to assemble collagen fibers so efficiently, just clone in that beef savory-peptide; we are on our way to a *Pichia* burger. May be the Phillips Petroleum Company's initial *Pichia*-SCP dream, will be realized after all.

I can see someone out there read *Pichia* genome, create knockout and knockin strains for every *Pichia* ORF, and study their impact on recombinant protein expression in a bioreactor! Thinking of bioreactors, I am a bit puzzled why *Pichia* has not yet been adopted for cultivation in wave bioreactors offered by GE Healthcare (http://www.wavebiotech.com/products/wave_bioreactor/system-500/index.html). Wave technology is a completely closed system with disposable bioreactor chamber, scalable to 500 L, easy to operate, and provides controllers for dissolved oxygen, pH, CO_2, O_2, and other optional modules. Designed for R&D, process development and cGMP production use, the Wave Bioreactor is gaining popularity for mammalian and insect cell cultivation and has recently been adopted for *S. cerevisiae* (155). Culture medium and cells only contact a presterile, disposable chamber called a Cellbag that is placed on a special rocking platform. The rocking motion of this platform induces waves in the culture fluid. These waves provide mixing and oxygen transfer, resulting in a perfect environment for cell growth. The bioreactor requires no cleaning or sterilization, providing the ultimate ease in operation and protection against cross-contamination.

With the expiry of the key *Pichia* patent, and presence of literally hundreds of *Pichia*-experts around the globe, many more industrial outfits will embrace *Pichia* and we can anticipate many more *Pichia* derived proteins and vaccines to be commercialized in the near future. May be we will see *Pichia* engineered to fix CO_2, as well as makes its own O_2, so that we would not have to worry about feeding methanol or oxygen to bioreactors—certainly a sigh of relief!

MEDIA COMPOSITIONS

Stock solutions (42)

10 × YNB: 13.4 g of yeast nitrogen base without amino acids (YNB) in 100 mL of water and filter sterilize.

500 × B: 20 mg D-biotin in 100 mL water and filter sterilize.

100 × H: 400 mg L-Histidine in 100 mL water (heat if necessary) and filter sterilize.

10 × D: 20 g D-glucose in 100 mL water. Autoclave or filter sterilize.

10 × GY: Mix 10 mL of glycerol with 90 mL water and filter sterilize.

10 × M: Mix 5 mL methanol with 95 mL water and filter sterilize.

Minimal media compositions (42)

MD: Mix 100 mL of 10 × YNB, 2 mL of 500 × B, and 100 mL of 10 × D with 800 mL autoclaved water (include 15 g bacto-agar for plates).

MM: Mix 100 mL of 10 × YNB, 2 mL of 500 × B, and 100 mL of 10 × M with 800 mL autoclaved water (include 15 g bacto-agar for plates).

MGY: Mix 100 mL of 10 × YNB, 2 mL of 500 × B, and 100 mL of 10 × Gy with 800 mL autoclaved water (include 15 g bacto-agar for plates).

Supplemental medium (42)

MDH: Mix 100 mL of 10 × YNB, 2 mL of 500 × B, 100 mL of 10 × D, and 10 mL 100 × H with 790 mL autoclaved water (include 15 g bacto-agar for plates).

MMH: Mix 100 mL of 10 × YNB, 2 mL of 500 × B, 100 ml of 10 × M, and 10 mL 100 × H with 790 mL autoclaved water (include 15 g bacto-agar for plates).

MGYH: Mix 100 mL of 10 × YNB, 2 ml of 500 × B, 100 mL of 10 × MGY, and 10 mL 100 × H with 790 mL autoclaved water (include 15 g bacto-agar for plates).

Complex media composition (42)

YPD: Dissolve 10 g bacto yeast extract, 20 g peptone, and 20 g glucose in water (also include 15 g bacto-agar for plates) and autoclave for 20 min.

YMPD: Dissolve 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 10 g of glucose in 1 L of water and autoclave.

YMPGY: Same as YMPD with the exception that 10 mL of 100% glycerol is used instead of 10 g glucose.

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Secretion media composition (42)

BMGY: Mix 100 mL of 1 *M* potassium phosphate buffer, pH 6, 100 mL of 10 × YNB, 2 mL of 500 × Biotin, and 10 mL of glycerol. Filter sterilize and add to an autoclaved solution of 10 g yeast extract and 20 g peptone in 788 mL water (15 g bacto-agar is included for plates),

BMMY: Same as BMGY, with the exception that 5 mL methanol is added in the place of 10 mL glycerol.

Note: Yeast extract and peptone in the above media can be replaced by 1% casamino acids.

The following buffers are used to adjust the pH of BMGY and BMMY to desired pH:

Phosphate buffer for pH range 5.7–8

Alanine–HCl buffer for pH range 2.8–3.6

Aconitic acid–NaOH buffer for pH range 3–5.7

Citrate buffer for pH range 3–6.2

Avoid succinate buffer, because succinic acid will repress induction of the *AOX1* promoter.

Fermentation Medium and Reagents

Basal salts medium (BSM) derived from Wegner (3)

For 1 L, mix together the following ingredients:

H_3PO_4 (85%) 26.7 mL

$CaSO_4 \cdot 2H_2O$ 0.93 g

K_2SO_4 18.2 g

$MgSO_4 \cdot 7H_2O$ 14.9 g

KOH 4.13 g

Glycerol 40 g

Water to 1 L

After autoclaving, adjust the pH to 5 with 28% NH_3 (concentrated ammonium hydroxide). It is normal that the medium becomes cloudy at pH 5. The added ammonium hydroxide serves as nitrogen source during fermentation.

FM21 basal salt media, derived from Wegner (3)

For 1 L, mix together the following ingredients:

H_3PO_4 (85%) 3.5 mL

$CaSO_4 \cdot 2H_2O$ 0.15 g

K_2SO_4 2.4 g

$MgSO_4 \cdot 7H_2O$ 1.95 g

KOH 0.65 g

Glycerol 40 g

Water to 1 L

FM22 basal salt media (121)

For 1 L, mix together the following ingredients:

KH_2PO_4 42.9 g

$CaSO_4 \cdot 2H_2O$ 18.2 g

$(NH_4)_2SO_4$ 5 g

K_2SO_4 14.3 g

$MgSO_4 \cdot 7H_2O$ 1.95 g

Glycerol 40 g

After autoclaving pH is approximately 4.5

PTM1 trace salts derived from Wegner (3)

Mix together the following ingredient in 1 L of water:

$CuSO_4 \cdot 5H_2O$ 6 g

NaI 0.08 g

$MgSO_4 \cdot H_2O$ 3 g

$Na_2MoO_4 \cdot 2H_2O$ 0.2 g

H_3BO_3 0.02 g

$CoCl_2$ 0.5 g

$ZnCl_2$ 20 g

$FeSO_4 \cdot 7H_2O$ 65 g

Biotin 0.2 g

H_2SO_4 5 mL

Water to a final volume of 1 L

Filter sterilize and add 4.35 mL/L to the autoclaved starting medium.

Note: There may be a cloudy precipitate upon mixing of these ingredients.

The precipitate does not have any adverse effect on growth of *Pichia*.

If precipitation is a concern, then PTM4 (see section titled "PTM4 Trace Salts"), which has lower amounts of copper, iron, zinc, and sulfuric acid may be used.

PTM4 trace salts (122)

Mix together the following ingredient in 1 L of water:

$CuSO_4 \cdot 5H_2O$ 2 g

NaI 0.08 g

$MnSO_4 \cdot H_2O$ 3 g

$Na_2 \cdot Na_2MoO_4 \cdot 2H_2O$ 0.2 g

H_3BO_3 0.02 g

$CoCl_2$ 0.5 g

$ZnCl_2$ 6.7 g

$FeSO_4 \cdot 7H_2O$ 21.6 g

Biotin 0.2 g

H_2SO_4 1.7 mL

Water to a final volume of 1 L

Filter sterilize and add 4.35 mL/L to the autoclaved starting medium.

5% (w/v) antifoam solution (AF) (122)

Dissolve 25 g of KFO™ 673 antifoam (food grade, KAOB Chemicals, Inc., WY, USA) in 500 mL water, filter sterilize, and add 2 mL/L to the starting medium. Additional amounts may be added as needed to eliminate foam during the fermentation.

GLOSSARY OF *P. PASTORIS* STRAINS

Y-11430-SC5 (wild type) (NRRL)

X-33 (wild type) Invitrogen

GS115 (*his4*)—this Mut^+ strain is also known as GTS115 (54)

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KM71 (*his4, aox1::SARG4*)—this is Mut^S strain (28)

PPF1 (*his4, arg4*) (28)

MC100-3 (*aox1::SARG4, aox2::HIS4, his4, arg4*)—this is Mut^- (44)

Protease-deficient strains

SMD 1168 (*his4*, *pep4*) (95)

SMD1165 (*his4*, *prB1*) (95)

SMD 1163 (*his4*, *pep4*, *prB1*) (95).

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