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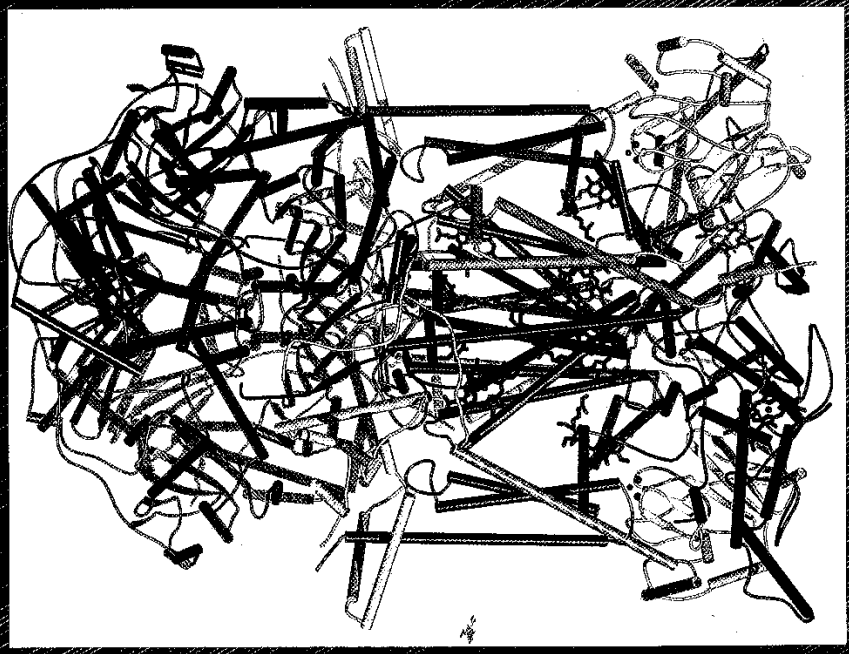
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Gene Regulatory Mechanisms



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Gene Regulatory Mechanisms

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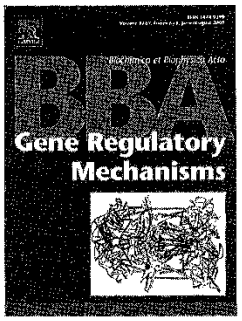
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Front cover image: Image of the X-ray structure of Cytochrome bc1 complex from the yeast Saccharomyces cerevisiae, kindly provided by Carola Hunte, University of Frankfurt.

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Identification of key DNA elements involved in promoter recognition by Mxr1p, a master regulator of methanol utilization pathway in *Pichia pastoris*

Balla Venkata Kranthi, Ritesh Kumar, Nallani Vijay Kumar, Desirazu N. Rao, Pundi N. Rangarajan *

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

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ABSTRACT

Mxr1p (methanol expression regulator 1) functions as a key regulator of methanol metabolism in the methylotrophic yeast *Pichia pastoris*. In this study, a recombinant Mxr1p protein containing the N-terminal zinc finger DNA binding domain was overexpressed and purified from *E. coli* cells and its ability to bind to promoter sequences of *AOXI* encoding alcohol oxidase was examined. In the *AOX1* promoter, Mxr1p binds at six different regions. Deletions encompassing these regions result in a significant decrease in *AOXI* promoter activity *in vivo*. Based on the analysis of *AOXI* promoter sequences, a consensus sequence for Mxr1p binding consisting of a core 5′ CYCC 3′ motif was identified. When the core CYCC sequence is mutated to CYCA, CYCT or CYCM (M = 5-methylcytosine), Mxr1p binding is abolished. Though Mxr1p is the homologue of *Saccharomyces cerevisiae* Adr1p transcription factor, it does not bind to Adr1p binding site of *S. cerevisiae* alcohol dehydrogenase promoter (*ADH2UAS1*). However, two point mutations convert *ADH2UAS1* into an Mxr1p binding site. The identification of key DNA elements involved in promoter recognition by Mxr1p is an important step in understanding its function as a master regulator of the methanol utilization pathway in *P. pastoris*.

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1. Introduction

The methylotrophic yeast *Pichia pastoris* is an excellent model organism for the study of eukaryotic gene expression and peroxisome biogenesis as well as production of recombinant proteins [1–4]. In *P. pastoris*, expression of a number of genes involved in methanol metabolism as well as peroxisome biogenesis is induced when methanol or fatty acids are used as the sole carbon source [2,3]. Among the methanol-inducible genes, the gene encoding alcohol oxidase I (*AOXI*), the first enzyme in methanol metabolism is expressed at very high levels and as a result *P. pastoris AOX1* promoter (P_{AOX1}) has become one of the most extensively used eukaryotic promoters for recombinant protein expression [1,2]. Despite its industrial importance, the mechanism by which methanol induces the transcription of genes involved in methanol utilization pathway is not well understood. Recently, a zinc finger transcription factor named Mxr1p (methanol expression regulator 1) was identified as the global regulator of genes involved in the methanol utilization pathway [5]. *P. pastoris* strains lacking Mxr1p are unable to grow on peroxisomal substrates such as methanol and oleic acid and expression of *AOXI* as well as several genes involved in the methanol utilization pathway is severely affected in the *mxr1*Δ strain [5]. Methanol-inducible expression of *AOXI* as well as several other genes of the methanol utilization pathway is severely affected in *mxr1*Δ strain [5]. A number of promoter sequences involved in methanol-inducible expression of P_{AOX1} have been recently identified and some of these harbour potential Mxr1p binding sites [6]. However, transcription factors binding to these sequences have not been biochemically characterized.

Mxr1p consists of a C_2H_2-type zinc finger in the amino terminus which shares 70% identity with the zinc finger DNA binding domain of *Saccharomyces cerevisiae* Adr1p (alcohol dehydrogenase II synthesis regulator), a transcription factor regulating the expression of genes involved in the metabolism of glycerol, ethanol and oleic acid [5]. In view of the high degree of homology between the DNA binding domains of Mxr1p and Adr1p, it was suggested that the Mxr1p and Adr1p may bind similar DNA sequences [5]. While the Adr1p binding sites are well characterized, the DNA sequence requirements for Mxr1p binding are not known. Electrophoretic mobility shift assays (EMSA) carried out with *P. pastoris* cell extracts overexpressing Mxr1p and a 243 bp P_{AOX1} DNA indicated the presence of an Mxr1p binding site between −415 and −172 bp. This region contains a sequence similar to that recognized by Adr1p suggesting that Mxr1p and Adr1p may recognize similar DNA sequences [5]. However, *in silico* analysis of P_{AOX1} for transcription factor binding sites revealed the presence of a putative Adr1p binding site between −577 and −571 bp [6]. Whether Mxr1p actually binds to any of these putative Adr1p binding

Abbreviations: Mxr1p, methanol expression regulator 1; AOXI, alcohol oxidase I; P_{AOX}, promoter of *AOXI*; DHAS, dihydroxyacetone synthase; PEX8, peroxin 8; MXRE, MXR response element; EMSA, electrophoretic mobility shift assay; Adr1p, alcohol dehydrogenase II synthesis regulator; HP0051, *Helicobacter pylori* C^5 cytosine methyltransferase

* Corresponding author. Tel./fax: +91 80 23601492.
E-mail addresses: pnr@biochem.iisc.ernet.in, pnrangarajan@yahoo.com (P.N. Rangarajan).

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sites in P_{AOX1} is not known. In this study, the N-terminal region of Mxr1p containing the DNA binding domain was overexpressed in *E. coli* cells and the ability of the purified recombinant protein to bind to *AOX1* promoter sequences (−940 to −1 bp) was evaluated by EMSA as well as DNase I foot printing. Such studies have led to the identification of six Mxr1p response elements in P_{AOX1} (P_{AOX1}-MXREs), each of which is of 20 bp in length and consists of a core 5′ CYCC/GGRG 3′ sequence. Interestingly, Mxr1p does not recognize the Adr1p binding sites present in the *S. cerevisiae* of alcohol dehydrogenase promoter upstream activation sequence 1 (*ADH2UAS1*), unless two adenine residues are mutated to cytosine.

2. Materials and methods

2.1. Synthesis of oligonucleotides and preparation of radiolabeled DNA probes

Oligonucleotides were purchased from Sigma-Aldrich Chemicals (Bangalore, India). DNA containing Adr1p binding site present in *S. cerevisiae ADH2* promoter from −237 to −215 bp [7–9] was generated by annealing the oligonucleotide 5′ GGGTCTCCAACTTA-TAAGTTGGAGACCC 3′ with its complement. ^{32}P-labeled DNA probes were generated by radiolabeling the 5′ ends of double stranded oligonucleotides with [γ-^{32}P]ATP and T4 polynucleotide kinase using standard molecular biology protocols [10]. After radiolabeling, unincorporated radionucleotides were removed by sephadex G-50 chromatography.

2.2. Preparation of recombinant Mxr1p

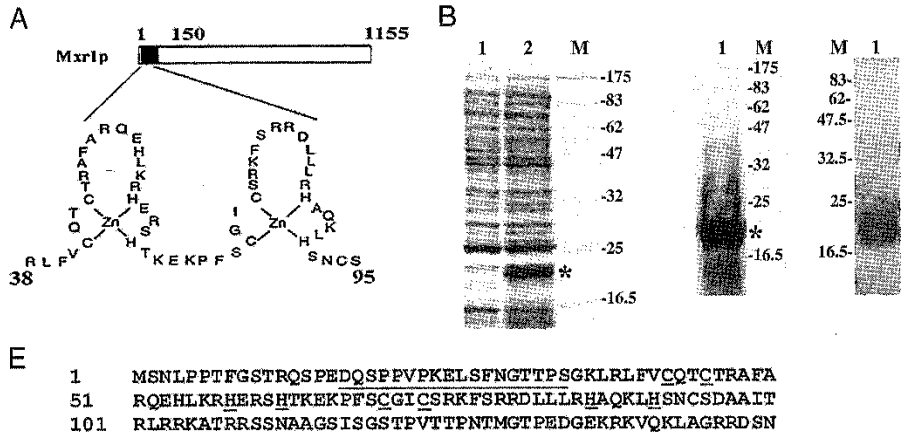
The cDNA encoding the first 150 N-terminal amino acids of Mxr1p was obtained by PCR amplification of *P. pastoris* genomic DNA using the primer pair 5′ CGCGGATCCATGAGCAATCTACCCCCAAC 3′ and 5′ CCCAAGCTTTCAATTTGAGTCCCGGCGGCC 3′ and cloned into BamHI and HindIII sites of pRSETA vector (Invitrogen, USA) using standard molecular biology protocols [10]. The restriction sites in the primers are underlined. The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS cells and grown at 37 °C until an A_{600} of 0.8 was reached. Expression of the recombinant protein was induced for 3 h by the addition of 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were harvested and resuspended in buffer A (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 10% glycerol and 10 mM β-mercaptoethanol) containing 10 mM imidazole. Cells were lysed by sonication and the lysate was centrifuged at 10,000 g at 4 °C for 30 min. The clear supernatant was loaded onto a Ni^{2+}-NTA agarose column (Qiagen, USA) equilibrated with buffer A. The column was washed with buffer A containing 50 mM imidazole and the recombinant protein was eluted sequentially with buffer A containing 100 mM and 300 mM imidazole. The 300 mM eluate fractions containing histidine-tagged recombinant Mxr1p protein were pooled, dialysed against buffer A and stored in aliquots at −80 °C. Western blot analysis of the purified protein was carried out with anti-histidine tag antibodies (Bangalore Genei, Bangalore, India). The recombinant protein was subjected to trypsin digestion and the tryptic peptides were purified by reverse phase HPLC. One of the purified peptides was sequenced using Procise™ protein sequencing system (Perkin–Elmer, Applied Biosystems, Foster, CA).

2.3. Electrophoretic mobility shift assay (EMSA)

Purified recombinant Mxr1p (30 nM/10 ng) was incubated with ^{32}P-labeled DNA probes (20,000 cpm, ~0.2–0.5 nM) in a 30 μl reaction containing buffer B (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.05% NP-40, 100 ng poly (dI-dC) and 6% glycerol) for 30 min at 4 °C. In some experiments, Mxr1p (~25–60 nM) was incubated first with excess amount of unlabeled oligonuceotides for 5 min prior to the addition of ^{32}P-labeled DNA probes. The reaction mixtures were subjected to electrophoresis at 4 °C on 5% non-denaturing polyacrylamide gel in a buffer containing 7 mM Tris–HCl (pH 7.4), 3 mM boric acid and 1 mM EDTA. The gels were dried and autoradiographed.

2.4. Measurement of the apparent dissociation constant

The apparent dissociation constant (K_d) of Mxr1p binding to *AOX1* promoter sequences was estimated as the protein concentration at which half of the radiolabeled DNA probe was shifted in an EMSA. EMSA was carried out with radiolabeled P_{AOX}*MXREs* (~0.5 nM) and varying amounts of Mxr1p in the presence of 100 ng of poly(dI-dC) per 30 μl reaction as described above. The intensity of shifted bands and free probes was measured in a PhosphorImager (Molecular Dynamics). All quantitations were done using ImageQuant software, version 5.0 (Molecular Dynamics). Experiments were repeated twice and the mean ± standard deviation was calculated. The K_d values were also calculated by non-linear regression using GraphPad Prism 5



E

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1    MSNLPPTFGSTRQSPEDQSPPVPKELSFNGTTPSGKLRLFVCQTCTRAFA
51   RQEHLKRHERSHTKEKPFSCGICSRKFSRRDLLLRHAQKLHSNCSDAAIT
101  RLRRKATRRSSNAAGSISGSTPVTTPNTMGTPEDGEKRKVQKLAGRRDSN
```

Fig. 1. Overexpression and purification of recombinant Mxr1p containing the first 150 N-terminal amino acids. (A) Schematic representation of Mxr1p overexpressed and purified from *E. coli* cells. The C_2H_2 zinc finger DNA binding domain located between amino acids 38 and 95 is indicated. (B) Coomassie blue-stained SDS gel pattern of lysates of recombinant *E. coli* cells cultured in the absence (lane 1) or presence (lane 2) of IPTG. (C) Mxr1p purified by Ni^{2+} affinity chromatography. Asterisk indicates Mxr1p. (D) Western blot analysis of recombinant Mxr1p with anti-Histidine tag antibodies. (E) Amino acid sequence of recombinant Mxr1p used in this study. The region homologous to Adr1p PAR [11–13] is underlined. The cysteine and histidine residues of Mxr1p zinc fingers which co-ordinate with the zinc atoms are also underlined.

Motif Exhibit 1059, 184 of 653

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Table 1
Oligonucleotides spanning the *P. pastoris AOX1* promoter.

Name	*AOX1* promoter region (bp)	Oligonucleotide sequence
940	−940 to −881	5′ AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCG**ACATCCACAG** 3′
940c		3′ TCTAGATTGTAGGTTTCTGCTTTCCAACTTACTTTGGAAAAACGGTAGGC**TGTAGGTGTC** 5′
890	−890 to −831	5′ **ACATCCACAG**GTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGG**ATACACTAGC** 3′
890c		3′ **TGTAGGTGTC**CAGGTAAGAGTGTGTATTCACGGTTTGCGTTGTCCTCCCC**TATGTGATCG**
840	−840 to −781	5′ **ATACACTAGC**AGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTC**CTCAACACCC** 3′
840c		3′ **TATGTGATCG**TCGTCTGGCAACGTTTGCGTCCTGGAGGTGAGGAGAAGAG**GAGTTGTGGG**
790	−790 to −731	5′ **CTCAACACCC**ACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTG**ATTGGAGCTCG** 3′
790c		3′ **GAGTTGTGGG**TGAAAACGGTAGCTTTTTGGTCGGGTCAATAACCCGAACT**AACCTCGAGC**
740	−740 to −681	5′ **TTGGAGCTCG**CTCATTCCAATTCCTTCTATTAGGCTACTAACACCATGAC**TTTATTAGCC** 3′
740c		3′ **AACCTCGAGC**GAGTAAGGTTAAGGAAGATAATCCGATGATTGTGGTACTG**AAATAATCGG** 5′
690	−690 to −631	5′ **TTTATTAGCC**TGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTA**TTTCCGAATG** 3′
690c		3′ **AAATAATCGG**ACAGATAGGACCGGGGGGACCGCTCCAAGTACAAACAAATA**AAAGGCTTAC**
640	−640 to −581	5′ **TTTCCGAATG**CAACAAGCTCCGCATTACACCCGAACATCACTCCAGATG**AGGGCTTTCTG** 3′
640c		3′ **AAAGGCTTAC**GTTGTTCGAGGCGTAATGTGGGCTTGTAGTGAGGTCTACT**CCCGAAAGAC** 5′
590	−590 to −531	5′ **GGGCTTTCTG**AGTGTGGGGTCAAATAGTT**TCATGTTCCCCAAATGGCCCA**AAA**CTGACAG** 3′
590c		3′ **CCCGAAAGAC**TCACACCCCAGTTTATCAAAGTACAAGGGGTTTACCGGGT**TTTGACTGTC** 5′
540	−540 to −481	5′ **AAACTGACAG**TTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGA**TCTCATCCAA** 3′
540c		3′ **TTTGACTGTC**AAATTTGCGACAGAACCTTGGATTATACTGTTTTCGCACT**AGAGTAGGTT**
490	−490 to −431	5′ **TCTCATCCAA**GATGAACTAAGTTTGGTTCGTTGAAATGCTAACGGCCAGT**TGGTCAAAAA** 3′
490c		3′ **AGAGTAGGTT**CTACTTGATTCAAACCAAGCAACTTTACGATTGCCGGTC**AACCAGTTTT** 5′
440	−440 to −381	5′ **TGGTCAAAAA**CAAACTTCCAAAAGTCGCCATACCGTTTGTCTTGTTTGGT**ATTGATTGAC** 3′
440c		3′ **ACCAGTTTT**CTTTGAAGGTTTTCAGCGGTATGGCAAACAGAACAAACCATA**ACTAACTG** 5′
390	−390 to −331	5′ **ATTGATTGAC**GAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCT**CTCTATCGCT** 3′
390c		3′ **TAACTAACTG**CTTACGAGTTTTTATTAGAGTAATTACGAATCGCGTCAG**AGAGATAGCGA** 5′
340	−340 to −281	5′ **CTCTATCGCT**TCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGGGAAACA**CCCGCT** 3′
340c		3′ **GAGATAGCGA**AGACTTGGGGCCACGTGGACACGGCTTTGCGTTTACCCCTTTG**TGGGCGA** 5′
287	−287 to −228	5′ ACCCGCTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGG 3′
287c		3′ TGGGCGAAAAACCTACTAATACGTAACAGAGGTGTAACATACGAAGGTTCTAAGACCACC 5′
227	−227 to −174	5′ GAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCT 3′
227c		3′ CTTATGACGACTATCGGATTGCAAGTACTAGTTTTAAATTGACAAGATTGGGGA 5′
173	−173 to −114	5′ ACTTGACAGCAATATATAAACAGAAGCAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCA 3′
173c		3′ TGAACTGTCGTTATATATTTGTCTTCCTTCGACGGGACAGAATTTGGAAAAAAAAATAGT 5′

Bgl II restriction site at −940 bp is underlined. Triangle indicates transcription start site (−114). Overlapping regions between two oligonucleotides are shown in bold face. Doublestranded oligonucleotides were radiolabeled at their 5′ ends by polynucleotide kinase and used in EMSA.

(Graphpad Software, Inc, San Diego, CA). The equation, $Y=(Yo-NS)(K^*)+NS$ was used, where Yo is the binding at time zero, NS is nonspecific binding at infinite time and K is the rate constant.

2.5. DNase I foot printing

Full length double stranded DNA was generated by annealing equimolar amounts of 5′ end labeled single stranded oligonucleotide with unlabeled complementary strand. These radiolabeled probes (20,000 cpm, ~0.5 nM) were incubated on ice with Mxr1p (60 nM/20 ng) and 100 ng of poly (dI-dC) in a 30 μl reaction mixture containing buffer B for 30 min. A 50 μl solution consisting of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added followed by 0.02 U of DNase I (Sigma-Aldrich, USA) and the samples were incubated at room temperature for 1 min. DNase I digestion was arrested by the addition of EDTA to a final concentration of 50 mM. Following deproteinization

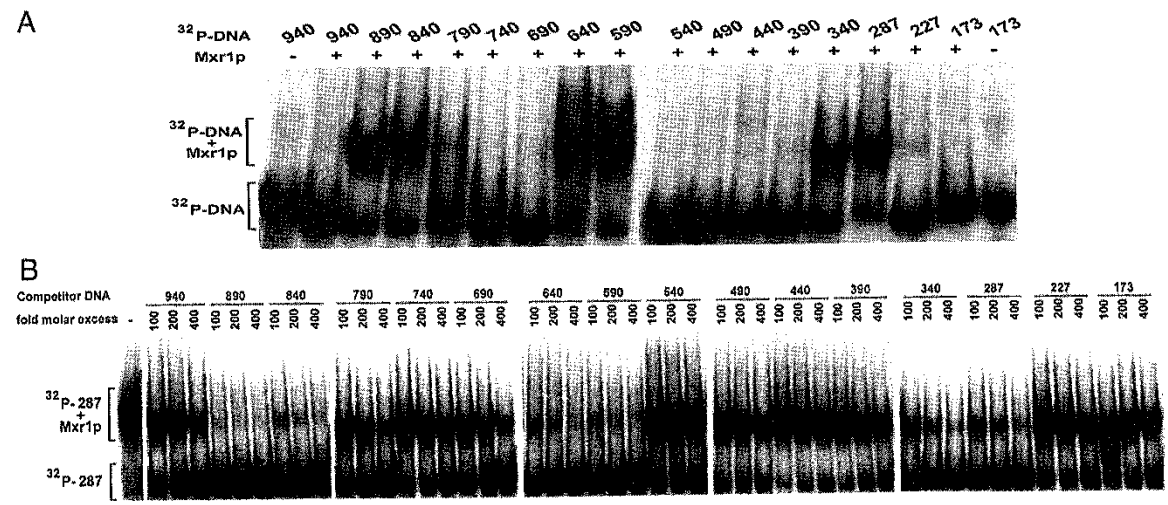


Fig. 2. Analysis of Mxr1p binding to P_{AOX1} DNA by EMSA. (A) Interaction of Mxr1p with ^{32}P-labeled P_{AOX1} oligonucleotides. Mxr1p forms a complex with six of the sixteen oligonucleotides. EMSA was carried out with radiolabeled probes (20,000 cpm, ~0.2–0.5 nM), 100 ng of (polydI-dC) and Mxr1p (30 nM). (B) Competition EMSA. Binding of Mxr1p (30 nM) to ^{32}P-labeled 287 oligonucleotide (20,000 cpm, ~0.5 nM) is outcompeted by 100–400 fold molar excess of unlabeled duplex oligonucleotides 890, 840, 640, 590, 340 and 287 but not 940, 790, 740, 540, 490, 440, 390, 227, and 173.

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and ethanol precipitation, the DNA was dissolved in a buffer containing 80% formamide, 50 mM Tris–borate, 1 mM EDTA, 0.01% xylene cyanol and 0.1% bromophenol blue. The DNA is denatured and then subjected to electrophoresis on 12% polyacrylamide gels containing 8 M urea. Foot prints were visualized by autoradiography and the foot print regions were identified by comparing their positions on the A + G sequence ladder.

2.6. Purification of Helicobacter pylori C^5 cytosine methyltransferase (HP0051)

The gene encoding *Helicobacter pylori* C^5 cytosine DNA methyltransferase, HP0051 (GenBank accession # AAD07117) was cloned into pET28a vector and transformed into *E. coli* BL21(DE3)pLysS cells. Conditions for overexpression and purification of HP0051 enzyme from *E. coli* extracts were similar to those employed for Mxr1p with minor modifications. The purified enzyme was stored in aliquots at −80 °C in 10 mM Tris–HCl, pH 8.0 containing 100 mM NaCl, 11 mM β-mercaptoethanol and 30% glycerol.

2.7. In vitro methylation of P_{AOX}MXREs using HP0051 enzyme

Methylation of P_{AOX}MXREs by HP0051 enzyme was monitored by the incorporation of tritiated methyl groups into DNA. Briefly, methylation assays were carried out in a reaction mixture containing 50 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol, 0.05 μM P_{AOX}MXREs, [*methyl*-^{3}H]-S-adenosylmethionine (66 Ci/mmol, GE Healthcare, India) and 0.5 μM purified HP0051 enzyme. After incubation of enzyme and substrate at 37 °C for 3 h, reactions were stopped by snap-freezing in liquid nitrogen and aliquots were transferred onto Whatman DE81 filter paper discs. Filters were washed three times (15 min each) with 200 mM ammonium bicarbonate solution equilibrated at 4 °C and once with 95% (v/v) ethanol. Filters were air dried, and the tritium content was determined in 3 ml of scintillation fluid (0.5% 2, 5-diphenyloxazole and 0.05% 1, 4-bis [2-(5-phenyl-oxazoyl)] benzene in 1:1 (v/v) 2-methoxy ethanol and toluene) in a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

For use in EMSA, ^{32}P-labeled double stranded DNA was methylated by HP0051 enzyme exactly as described above except

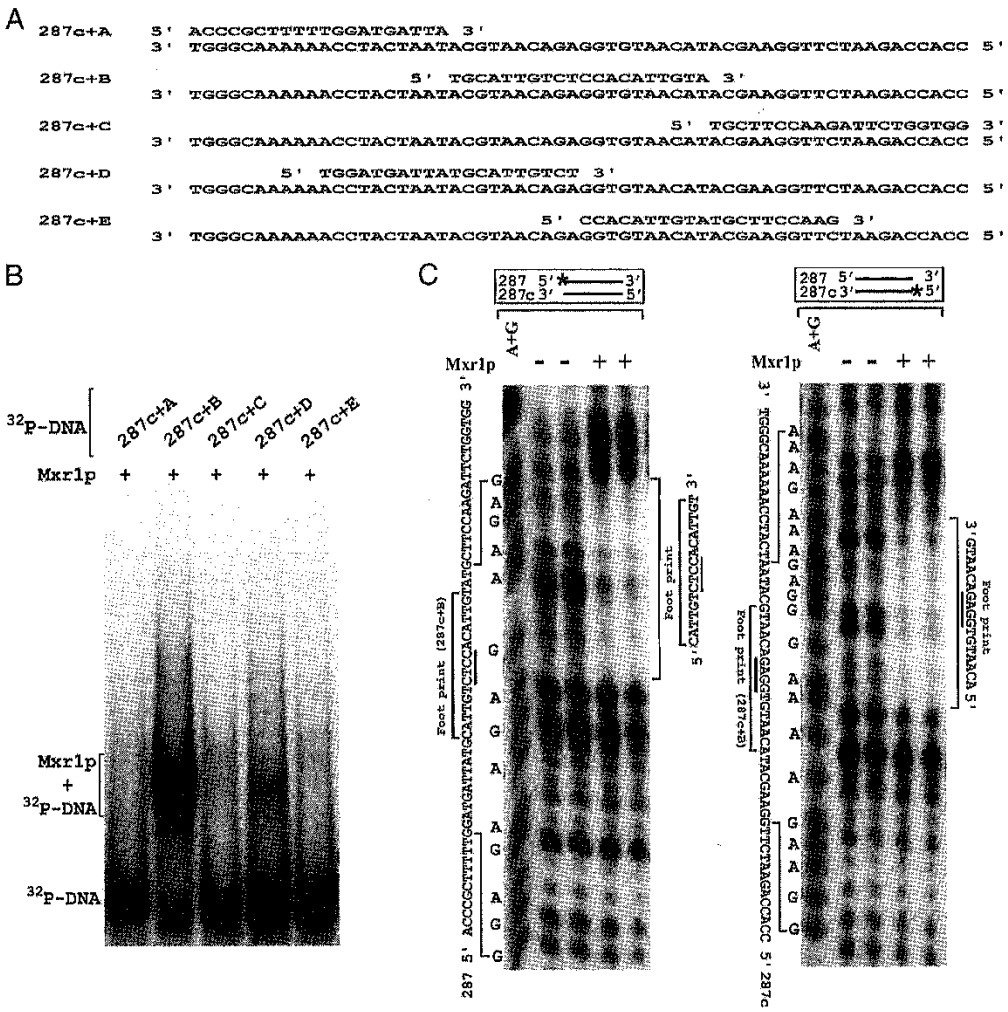


Fig. 3. Identification of P_{AOX1}MXRE in the 287 oligonucleotide by EMSA and DNase I foot printing. (A) Nucleotide sequence of partially double stranded oligonucleotides generated for use in EMSA. (B) Binding of Mxr1p to partially double stranded, radiolabeled oligonucleotides (20,000 cpm, ~0.15–0.20 nM) and Mxr1p (30 nM) as evaluated by EMSA. (C) DNase I foot print generated by Mxr1p in the sense (287) and antisense (287c) strands of 287 double stranded DNA (60 bp) is shown. Reactions were carried out in duplicate, in the absence (−,−) or presence (+,+) of 60 nM Mxr1p as described in Materials and methods. The double stranded DNA carrying radiolabel at the 5′ end of one of the DNA strands (asterisk) is shown schematically. The core sequence 5′ CTCC 3′ (in 287) and its complement, 5′ GGAG 3′ (in 287c) within the foot print are underlined (see text for details).

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that [*methyl*-^{3}H]-S-adenosylmethionine was replaced by 10 μM unlabeled S-adenosylmethionine. Following methylation, DNA was deproteinized, ethanol precipitated, redissolved in water and used in EMSA.

3. Results and discussion

3.1. Mxr1p binds at six different regions in P_{AOXI}

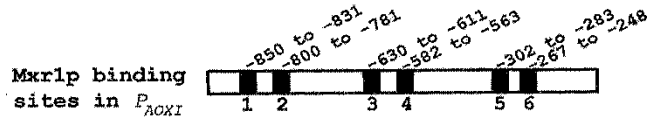
Mxr1p is a large protein of 1155 amino acids with an N-terminal zinc finger DNA binding domain located between amino acids 38 and 95 [5]. Our attempts to overexpress a recombinant Mxr1p protein containing only the zinc finger domain (amino acids 38–95) were not successful. However, Mxr1p containing 150 N-terminal amino acids could be expressed at high levels in *E. coli* cells and purified as a histidine-tagged protein (Figs. 1A–D). The recombinant protein was identified as Mxr1p by amino acid sequence analysis of one of the tryptic peptides (data not shown). This recombinant protein, referred to as Mxr1p in the rest of this study, contains a region homologous to the proximal accessory region (PAR) of Adr1p (Fig. 1E), in addition to the zinc finger domain [11–13].

To identify Mxr1p binding sites in P_{AOXI}, sixteen different double stranded oligonucleotides spanning the P_{AOXI} region between −940 and −1 were synthesized (Table 1) and their ability to bind to Mxr1p was examined by EMSA. Of the sixteen oligonucleotides, only six (890, 840, 640, 590, 340 and 287) generated a complex with Mxr1p (Fig. 2A). In competition experiments, only the duplex oligonucleotides 890, 840, 640, 590, 340 and 287 which bind to Mxr1p (Fig. 2A) efficiently outcompete Mxr1p binding to ^{32}P-labeled 287, when used as unlabeled competitors (Fig. 2B) indicating that Mxr1p binding to these DNA sequences is very specific.

3.2. Identification of minimal Mxr1p binding sites in P_{AOXI}

To identify the minimal Mxr1p binding sites within the six P_{AOXI} oligonucleotides, partially double stranded oligonucleotides

A



B

Deletion mutant[a]	Relative P_{AOXI} activity[a]	deleted region in P_{AOXI}[a]	Mxr1p binding site[b]
dHSF1	60%	Δ-805 to -798	2
d2	33%	Δ-643 to -597	3
dAdr1	30%	Δ-576 to -570	4
d5	40%	Δ-322 to -264	5,6
dMat1-MC	42%	Δ-264 to -260	6
d6	45%	Δ-253 to -224	6

[a] Hartner *et al.*, (6); [b] This study.

C



Fig. 4. Correlation between Mxr1p binding sites identified in this study and DNA sequences essential for P_{AOXI} function *in vivo*. (A) Schematic representation of the *AOXI* promoter and location of the six Mxr1p binding sites identified in this study. (B) Effect of deletion of different regions of *AOXI* promoter on methanol-inducible P_{AOXI} activity *in vivo* as reported by Hartner et al. [6]. The six Mxr1p binding sites (P_{AOX}MXREs) identified in this study (last column) are present within these deleted regions. (C) Nucleotide sequence of *AOXI* promoter and location of the P_{AOX}MXREs in *AOXI* promoter. The conserved 5′ CYCC/GGRG 3′ motifs in the MXREs are underlined. The promoter deletions which affect P_{AOXI} function *in vivo* as reported by Hartner et al. [6] are shown by broken lines and arrows.

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were generated by annealing the antisense strands with short, sense strand oligonucleotides. For example, partially double stranded regions were generated in the P_{AOX1} region between −287 and −228 by annealing the 60 bp long antisense strand (287c) with five different sense strand oligonucleotides, each of which is of 20 bp in length (Fig. 3A). The five different partially double stranded oligonucleotides thus generated (287c+A–287c+E) were radiolabeled and used in EMSA. Interestingly, only one of them (287c+B) generated a prominent complex with Mxr1p (Fig. 3B) indicating the presence of an Mxr1p binding site within this 20 bp region. A faint Mxr1p–DNA complex was obtained with 287c+D and this may be due to the presence of a 10 bp region that is common between 287c+D and 287c+B. The Mxr1p binding site within double stranded 287+287c oligonucleotide was also identified by DNase I foot printing. A foot print was generated by Mxr1p in the 287c+B region in the sense as well as antisense strands (Fig. 2C). Thus, using a combination of EMSA and DNase I foot printing, Mxr1p binding sites were identified in all the remaining five oligonucleotides (Fig. 4A, Supplementary Figs. S1–S5).

3.3. Deletion of Mxr1p binding sites in P_{AOX1} reduces promoter activity in vivo

To examine whether the Mxr1p binding sites identified in P_{AOX1} have a role in P_{AOX1} function *in vivo*, we compared their location with that of cis-acting elements and putative transcription factor binding sites identified recently by Hartner et al. [6]. Such a comparison revealed a significant overlap between the Mxr1p binding sites identified in this study and putative binding sites for transcription factors such as HSF1, Mat1-MC and Adr1p reported by Hartner et al. [6]. Further, a number of promoter deletions which result in a significant decrease in P_{AOX1} activity *in vivo* involved deletions within Mxr1p binding sites (Fig. 4B). For example, the d2 deletion between −643 and −597, whose deletion results in a 67% decrease in P_{AOX1} activity, includes Mxr1p binding site 3 (Fig. 4B). Similarly, d5, dMat1-MC and d6 deletions include deletions within Mxr1p binding sites 5 and 6 while dHSF1 and dAdr1 deletions involve deletions within Mxr1p binding sites 2 and 4 respectively (Fig. 4B). In view of the strong correlation between deletions within Mxr1p binding sites and decrease in P_{AOX1} function, we have named the six P_{AOX1} Mxr1p binding sites identified in this study as P_{AOX1} Mxr1p response elements (P_{AOX}MXREs) (Figs. 4B, C).

3.4. Identification of a conserved motif in P_{AOX}MXREs

To further characterize the P_{AOX}MXREs, six different oligonucleotides of 20 bp length referred to as P_{AOX}MXRE1–P_{AOX}MXRE6 were synthesized (Fig. 4C). The 20 bp region was chosen based on the results of EMSA with partially double stranded oligonucleotides wherein the 287c+B partially double stranded DNA, which contains a 20 bp double stranded region was found to bind Mxr1p very efficiently (Fig. 3B). When used in EMSA, all of the six P_{AOX}MXREs generated a complex with Mxr1p (Fig. 5A). Analysis of the nucleotide sequence of the P_{AOX}MXREs revealed that the sequence 5′ CYCC 3′ (5′ GGRG 3′ in the complementary strand) is conserved in all the six P_{AOX1}-MXREs (Fig. 4C). A single point mutation within this conserved motif (5′ CYCC 3′ to 5′ CYCA 3′) abrogated Mxr1p binding, indicating a critical role for this sequence in Mxr1p–DNA interactions (Fig. 5B). The importance of this conserved motif in Mxr1p binding was further confirmed by DNA methylation studies employing a recombinant *Helicobacter pylori* C^5 cytosine methyltransferase (HP0051), which specifically recognizes the 5′ GGRG 3′ sequence in the double stranded DNA and methylates the terminal cytosine residue on the opposite strand within this motif (Supplementary Fig. S6, R.K. and D.N.R. unpublished data). Methylation of a specific cytosine residue within the core 5′ CYCC 3′ motif of P_{AOX}MXREs by HP0051 enzyme was confirmed by an *in vitro* methylation reaction using [^{3}H]-S-adenosylmethionine as methyl group donor. Except P_{AOX}MXRE5, all other P_{AOX}MXREs were methylated with approximately the same efficiency by HP0051 enzyme (Supplementary Fig. S6). However, P_{AOX}MXRE4-M1, P_{AOX}MXRE6-M1, which carry C to A point mutation within the 5′ CYCC 3′ motif, were not methylated by HP0051 enzyme confirming the specificity of methylation reaction (Supplementary Fig. S6). In EMSA, Mxr1p binding to methylated P_{AOX}MXREs was significantly reduced (Fig. 6A) confirming a critical role for the cytosine residue within the 5′ CYCC 3′ motif in Mxr1p binding. Since methylcytosine as well as thymine carry the bulky methyl group at position 5 of the pyrimidine ring, we synthesized P_{AOX}MXRE4-M2 and P_{AOX}MXRE6-M2 oligonucleotides which carry C to T point mutation within the 5′ CYCC 3′ motif and both these mutants do not bind to Mxr1p (Fig. 6B). These results are summarized in Fig. 6C. Based on the alignment of the P_{AOX}MXREs, we have derived a core consensus sequence for Mxr1p binding (Fig. 6D). Further, we calculated the apparent dissociation constant (K_d) for Mxr1p binding to three of the six *P_{AOX}MXREs* and the K_d values were found to be in the range of 50–150 nM (Fig. 7). In comparison, the K_d values reported for Adr1p-*ADH2UAS1* interactions are in the range of

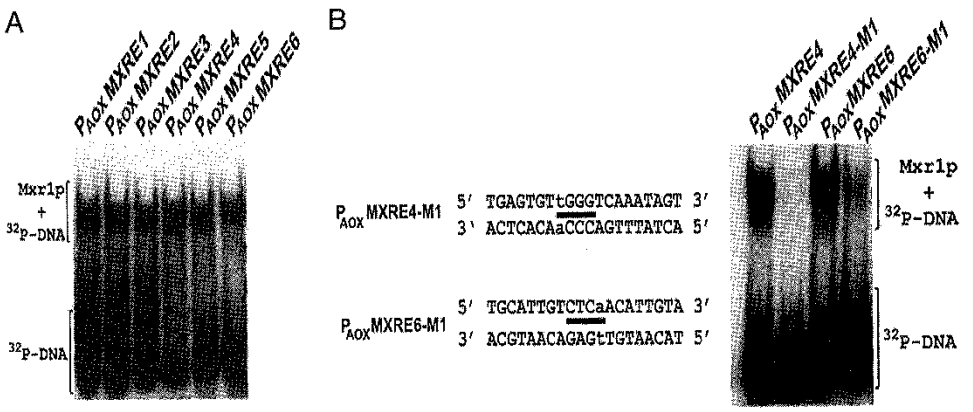


Fig. 5. Mxr1p binding to P_{AOX}MXREs as analyzed by EMSA. (A) Mxr1p (30 nM) binds to radiolabeled P_{AOX}MXREs (30,000 cpm, ~0.15–0.20 nM) in presence of 100 ng of poly (dI-dC). (B) Radiolabeled P_{AOX}MXREs (30,000 cpm, ~0.15–0.20 nM) carrying point mutations within the conserved 5′ CYCC 3′ motif (P_{AOX}MXRE4-M1, P_{AOX}MXRE6-M1) do not bind to Mxr1p (60 nM). The mutated nucleotides are indicated in lower case. The conserved core motif is underlined.

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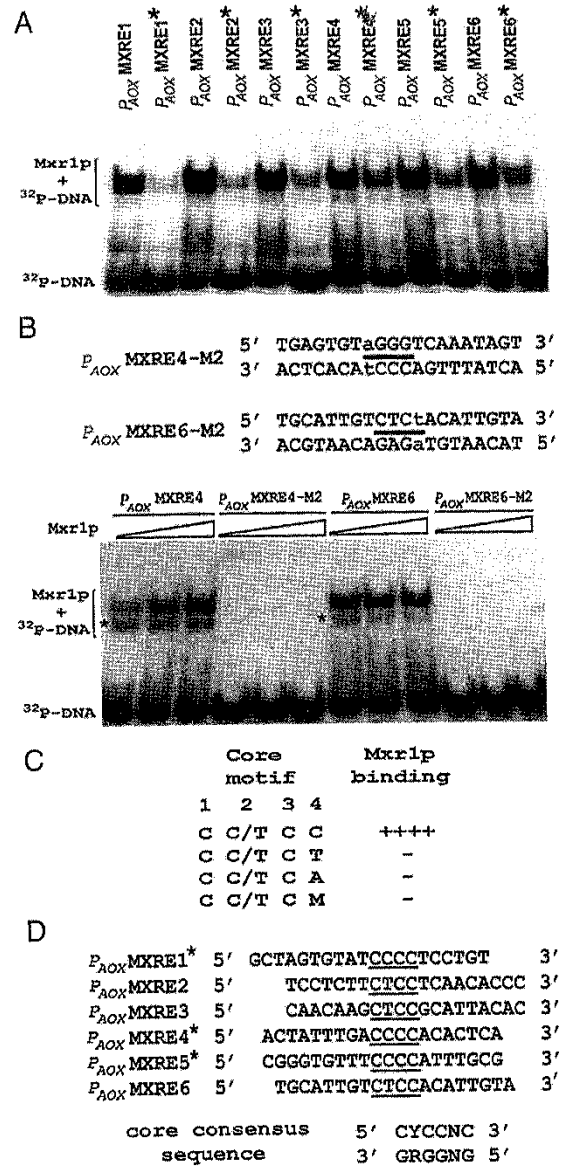


Fig. 6. Effect of methylation or mutation of a cytosine residue in the core motif of P_{AOX}MXREs on Mxr1p binding. (A) EMSA was carried out with radiolabeled, unmethylated as well as methylated (asterisk) P_{AOX}MXREs (30,000 cpm, ~0.15–0.25 nM) and Mxr1p (30 nM) as described in Materials and methods. (B) Effect of mutation of cytosine to thymine within the conserved 5′ CYCC 3′ motif of P_{AOX}MXREs on Mxr1p binding as evaluated by EMSA. The mutated nucleotides are indicated in lower case. The conserved core motif is underlined. Asterisk indicates complex formation by Mxr1p molecules which are not full length. These may arise due to incomplete translation or proteolytic degradation during isolation, purification and storage. (C) Summary of the mutagenesis and methylation studies. M is 5-methyl cytosine. (D) Alignment of the nucleotide sequences of P_{AOX}MXREs and derivation of a core consensus sequence for Mxr1p binding.

2–9 nM (7), suggesting that Mxr1p binds DNA with an affinity lower than that of Adr1p.

3.5. *Analysis of Mxr1p binding to S. cerevisiae alcohol dehydrogenase promoter upstream activation sequence 1 (ADH2UAS1)*

Mxr1p is considered to be a homologue of Adr1p [5,6] and therefore it was of interest to examine whether Mxr1p binds to Adr1p binding sites. An oligonucleotide containing the *S. cerevisiae ADH2*-UAS1 which harbours an Adr1p binding site was synthesized (Fig. 8A) and its ability to outcompete Mxr1p binding to radiolabeled P_{AOX}MXRE6 was evaluated by EMSA. The results presented in Fig. 8B indicate that *ADH2UAS1* failed to outcompete Mxr1p binding to ^{32}P-labeled P_{AOX}MXRE6. Increasing the length of *ADH2UAS1* to 38 bp such that the 5′ CTCC 3′ core motif is flanked by 9 bp, did not restore Mxr1p binding to *ADH2UAS1* (Fig. 8C). Alignment of the nucleotide sequences of Adr1p and Mxr1p binding sites indicated a key nucleotide difference between these two binding sites (Fig. 9A). We hypothesized that mutation of an adenine residue in the Adr1p monomer binding site of *ADH2UAS1* to cytosine should restore Mxr1p binding to *ADH2UAS1* (Fig. 9A). The results presented in Fig. 9B indicate that mutation of adenine to cytosine does restore Mxr1p binding to *ADH2UAS1*. Thus, despite extensive homology in the DNA binding domains and DNA recognition sequences of Mxr1p and Adr1p, the target gene specificity of these two zinc finger proteins is different.

In the case of Adr1p, the amino acid residues Arg115, His118 and Arg121 present in −1, +3, and +6 positions of the first zinc finger are known to contact the bases 3′ GA/GG 5′, of the core recognition motif (11). In the second zinc finger, only Arg143 present in the −1 position makes contact with the 5′ G residue of the core motif (11). Comparison of the zinc finger motif of Adr1p with that of Mxr1p indicates that all the amino acid residues mentioned above are also conserved in the Mxr1p zinc fingers (Supplementary Fig. S7A). Therefore, it is not surprising that both Adr1p and Mxr1p recognize the same core nucleotide quartet (5′ GGRG 3′). In addition to the two zinc fingers, a 20-amino acid domain referred to as the proximal accessory region (PAR) present N-terminal to the first zinc finger is also essential for high affinity DNA binding of Adr1p (12, 13). The Adr1p PAR exhibits 32% identity with a homologous region in Mxr1p and a number of amino acid residues within the PAR that are critical for DNA binding are conserved between these two proteins (Supplementary Fig. S7B). Further, in Adr1p PAR, Pro97 executes a turn so that Arg95, Gly94, Lys100 and Leu101 can contact the DNA (13). All these residues are also conserved in Mxr1p except Arg95 which is replaced by Thr31 in Mxr1p (Supplementary Fig. S7B). Since Mxr1p does not bind to *ADH2UAS1*, it will be interesting to examine whether T31R mutation in the putative PAR domain of Mxr1p will restore its binding to *ADH2UAS1*. Since Mxr1p binds to DNA with an affinity lower than that of Adr1p (Fig. 7), it is possible that amino acid differences in the PAR domains of Mxr1p and Adr1p as reported in this study, are responsible not only for altered DNA binding specificity but also weak affinity of Mxr1p for MXREs. Thus, it will be interesting to examine whether mutations in the putative PAR of Mxr1p affect the DNA binding specificity or DNA binding affinity or both.

Thus far, two putative Mxr1p binding sites have been reported in P_{AOXI}. Based on EMSA carried out with *P. pastoris* cell extracts overexpressing Mxr1p and a 243 bp DNA probe, an Mxr1p binding site was identified between −415 to −172 bp [5]. We now demonstrate that this region actually harbours two MXREs, P_{AOX}MXREs 5 and 6 (Fig. 4C). In another study, based on *in silico* analysis of P_{AOXI} for potential transcription factor binding sites, a putative Adr1p binding site was identified between −577 and −571 bp [6]. We now demonstrate that this region harbours P_{AOX}MXRE4. In addition, we have identified three more MXREs within P_{AOXI} (Fig. 4). The *AOXI* promoter deletion studies of Hartner et al. indicate that deletions encompassing five of the six P_{AOX}MXREs result in a significant decrease in P_{AOXI} function *in vivo* [6]. Based on the results presented in this study, it is now possible to carry out specific site-directed mutagenesis studies so that the effect of loss of Mxr1p binding to each of the six P_{AOX}MXREs on AOXI promoter function can be studied. It is likely that synergistic interactions amongst Mxr1p molecules binding to multiple P_{AOX}MXREs play a key role in the

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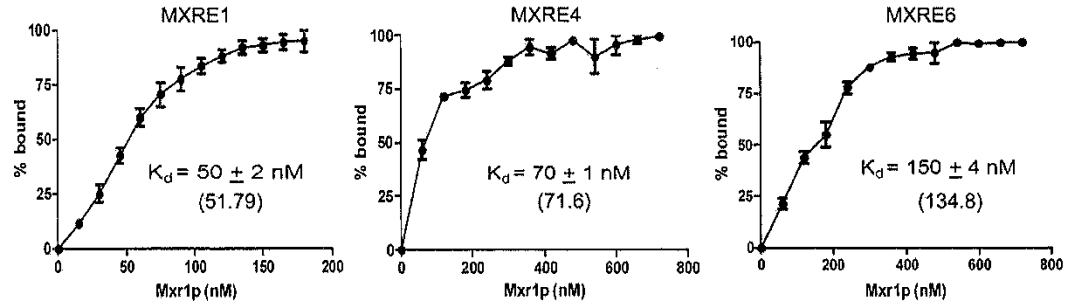


Fig. 7. Determination of K_d. EMSA reactions were carried out in the presence of 0.3–0.5 nM radiolabeled P_{AOX}MXREs and varying amounts of Mxr1p as indicated in the presence of 100 ng of poly dI-dC. In order to obtain K_d values of Mxr1p–P_{AOX}MXRE interactions, the percentage of radiolabeled DNA–Mxr1p complex was quantitated and plotted against the quantity of Mxr1p (nM) used. The data is the average of two independent saturation binding experiments and the error bars represent standard deviation of the mean. The numbers in parentheses represent K_d values calculated by non-linear regression using GraphPad Prism (for details, see Materials and methods).

high level of methanol-inducible expression of P_{AOX1}. Although Mxr1p appears to be the major regulator of P_{AOX1}, the fact that mutations and deletions in P_{AOX} outside the P_{AOX}MXREs also affect P_{AOX1} activity [6] indicate that cis-acting elements and transcription factors other than P_{AOX}MXREs and Mxr1p may also play a key role in the regulation of *AOX1* expression.

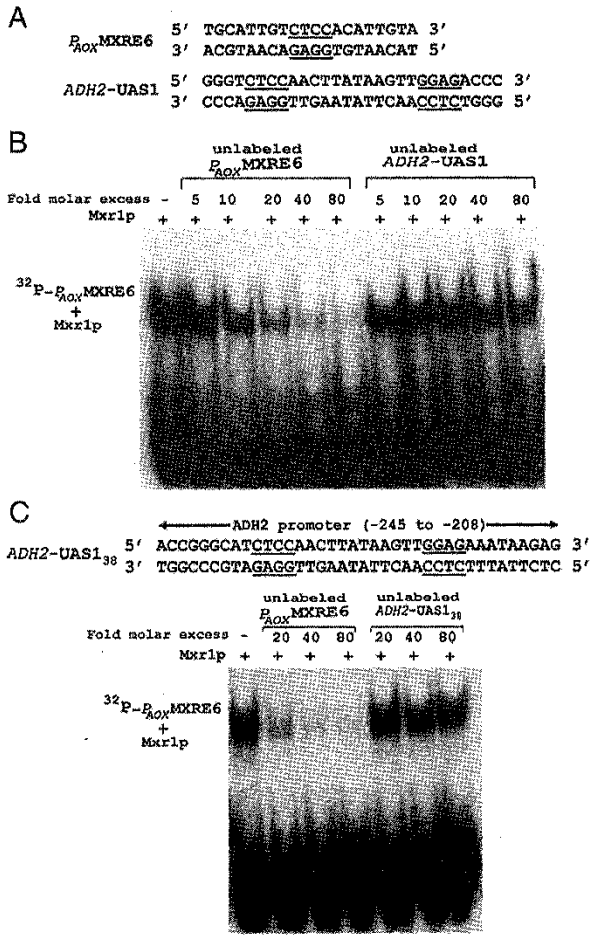


Fig. 8. Analysis of Mxr1p binding to *S. cerevisiae ADH2UAS1* by EMSA. (A) Nucleotide sequence of P_{AOX}MXRE6 and *ADH2UAS1*. The core CYCC motifs are underlined. (B) Competition EMSA was carried out with 0.5 nM radiolabeled probe and 30 nM Mxr1p. Unlabeled P_{AOX}MXRE6 but not *ADH2UAS1* competes out binding of Mxr1p to ^{32}P-labeled P_{AOX}MXRE6. (C) Effect of increasing the length of *ADH2UAS1* on Mxr1p binding as analyzed by competition EMSA.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbagrm.2009.05.004.

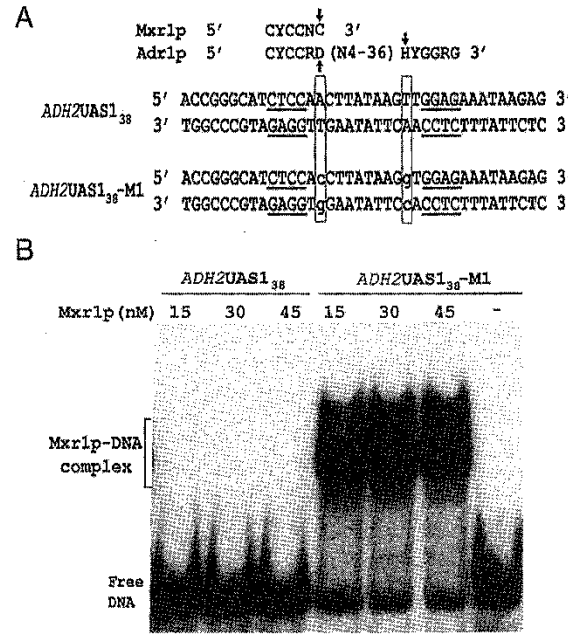


Fig. 9. Conversion of an Adr1p binding site into an Mxr1p binding site. (A) Comparison of consensus DNA sequences for the binding of Adr1p (9) and Mxr1p. Key nucleotide differences are indicated by arrows. Nucleotide sequence of the *ADH2UAS1*$_{38}$ corresponding to the *S. cerevisiae ADH2* promoter region between −245 and −208 bp is compared with that of the mutant oligonucleotide, *ADH2UAS1*$_{38}$-M1. The nucleotide differences are boxed and the mutated bases are indicated in lower case. The core 5′ CYCC 3′ motif is underlined in all the sequences. IUB nucleotide codes used are: H:A/C/T; D:A/G/T; Y:C/T; R:A/G; N:A/G/C/T. (B) Analysis of Mxr1p binding to ^{32}P-labeled *ADH2UAS1*$_{38}$ and *ADH2UAS1*$_{38}$-M1 by EMSA.

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Michael C. Flickinger
Golden LEAF Biomanufacturing Training and Education Center (BTEC),
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CONTRIBUTORS

Muhammad Aasim, Downstream Bioprocessing Laboratory, School of Engineering and Science, Jacobs University, Bremen, Germany, *Expanded Bed Chromatography, Surface Energetics of Biomass Deposition*

P. Marlene Absher, University of Vermont, College of Medicine, Colchester, Vermont, *Enrichment and Isolation Techniques for Animal Cell Types*

Luis A. Actis, Miami University, Oxford, Ohio, *Plasmid DNA Replication*

Myriam Adam, Laboratory of Cellular Biotechnology, Institute of Bioengineering, Faculty of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland, *Transient Gene Expression in Mammalian Cells*

Michael Adams, University of Georgia, Athens, Georgia, *Enzymes, Extremely Thermophilic*

S. Robert Adamson, Genetics Institute, Andover, Massachusetts, *Cell Stability, Animal*

Patrick von Aderkas, Graduate Centre for Forest Biology, University of Victoria, Victoria, Canada, *Conifers, Culture and Genetic Engineering*

Wolfgang Aehle, Genencor, A Danisco Division, Palo Alto, California; Brain Ag, Corporate Development, Zwingenberg, Germany, *Proteolytic Cleavage, Reaction Mechanisms*

Oscar Aguilar, Centro de Biotecnología Tecnológico de Monterrey, Monterrey, Mexico, *Expanded Bed Chromatography, Surface Energetics of Biomass Deposition*

Mattias Ahnfelt, GE Healthcare Bio-Sciences AB, Uppsala, Sweden, *High-Throughput Technologies in Bioprocess Development*

Kazuo Aisaka, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, *Cholesterol Oxidase*

Hiroyuki Akatsuka, Tanabe Seiyaku Co., Ltd., Osaka, Japan, *Diltiazem Synthesis*

Mohamed Al-Rubeai, University College Dublin, Belfield, Dublin, Ireland, *Apoptosis; Cell Cycle, Importance in Bioprocesses; Cell Cycle Synchronization*

Andrés-Rafael Alcántara, Facultad de Farmacia, Universidad Complutense, Madrid, Spain, *Enzyme-Catalyzed Synthesis of Nonnatural or Modified Nucleosides*

Marcos Almendros, Servicio de Biotransformaciones Industriales, Parque Científico de Madrid, Madrid, Spain, *Enzyme-Catalyzed Synthesis of Nonnatural or Modified Nucleosides*

Arie Altman, Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel, *Micropropagation of Plants*

Subbiah Alwarappan, Nanobioengineering and Bioelectronics Lab, Florida International University, Miami, Florida, *Nanomaterials Incorporated Bioelectronics*

Teruo Amachi, Kyoto University, Kyoto, Japan, *β-Galactosidase*

Ashraf Amanullah, Oceanside Process Research & Development, Genentech. Inc, Oceanside, California, *Animal Cell Culture Media*

Makhlouf Amoura, UPMC Univ Paris 6, CNRS and Collège de France, Chimie de la Matière Condensée de Paris, Paris, France, *Biocers, Industrial Applications*

Akinori Ando, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, *Oils, Microbial Production*

Silvana Andreescu, Clarkson University, Potsdam, New York, *Biosensors, Toxicity Monitoring*

Giuseppina Andreotti, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Napoli, Italy, *Enzymatic Transglycosylation*

Graham Andrews, MMBD Consulting, Gresham, Oregon, *Bioreactors, Gas Treatment*

P. Anthony, University of Nottingham, School of Biosciences, Sutton Bonington Campus, Loughborough, United Kingdom, *Plant Protoplasts*

Fabienne Anton, Institut für Technische Chemie, Gottfried Wilhelm Leibniz Universität Hannover, Hannover, Germany, *Fluorescence Techniques for Bioprocess Monitoring*

William Apel, Idaho National Engineering and Environmental Laboratory, Idaho Falls, Idaho, *Bioreactors, Gas Treatment*

Hazel Aranha, GAEA Resources Inc., Northport, New York, *Virus Retentive Filters*

Claude Artois, SmithKline Beecham Biologicals, Rixensart, Belgium, *Current Good Manufacturing Practice (CGMP) Compliance for Production Rooms*

Alahari Arunakumari, Medarex, Inc., Bloomsbury, New Jersey, *Inoculum Expansion Methods, Recombinant Mammalian Cell Lines*

Yasuhisa Asano, Toyama Prefectural University, Toyama, Japan, *D-Aminopeptidase and Alkaline D-Peptidase; Opine Dehydrogenase, Secondary Amine Dicarboxylic Acids; Phenylalanine Dehydrogenase*

Yasuhisa Asano, Mitsubishi Chemical Co., Yokohama, Japan, *D-Malate*

Yoshiro Ashina, Nitto Chemical Industry Co., Ltd., Tokyo, Japan, *Nitrile Hydratase*

J.A. Atwood, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications*

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John G. Auniņš, Merck Research Laboratories, West Point, Pennsylvania, *Viral Vaccine Production in Cell Culture*

Hans Axelsson, Alfa Laval AB, Tumba, Sweden, *Cell Separation, Centrifugation*

Diana C. S. Azevedo, Federal University of Ceará, Fortaleza-CE, Brazil, *Adsorption in Simulated Moving Beds*

Heino Büntemeyer, Institute of Cell Culture Technology, University of Bielefeld, Bielefeld, Germany, *Off-Line Analysis in Animal Cell Culture*

P. K. Bachmann, Philips Research Laboratories, Aachen, Germany, *Luminescent Materials, Biological Applications*

Euiwon Bae, School of Mechanical Engineering, Purdue University, West Lafayette, Indiana, *Biosensors, Foodborne Pathogen Detection*

Lucia Baldi, Laboratory of Cellular Biotechnology, Institute of Bioengineering, Faculty of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland, *Transient Gene Expression in Mammalian Cells*

Richard H. Baltz, CognoGen Enterprises, Indianapolis, Indiana, *Mutagenesis*

A. Baradarajan, Indian Institute of Technology Madras, Chennai, India, *Dextran, Microbial Production*

H.S.C. Barbosa, Center of Chemistry, University of Minho, Campus de Gualtar, Braga, Portugal, *Plasmid Purification, Therapeutic Applications*

José Luis Barredo, R&D Biology, Antibióticos S. A., León, Spain, *Cephalosporin Production by Fungal Metabolic Engineering*

Raquel Barrena, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain, *Dehydrogenase*

Amaro G. Barreto Jr., Escola de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro-RJ, Brazil, *Adsorption in Simulated Moving Beds*

Amarjeet S. Bassi, University of Western Ontario, Faculty of Engineering, London, Ontario, *Biosensors, Environmental*

Carl A. Batt, Cornell University, Ithaca, New York, *Bionanofabrication of Nanostructures and Functional Materials*

Hans Becker, Phytochemie Universität des Saarlandes, Saarbrücken, Germany, *Bryophyte In vitro Cultures, Secondary Products*

Leo A. Behie, Pharmaceutical Production Research Facility (PPRF), Schulich School of Engineering, University of Calgary, Alberta, Canada, *Neural Stem Cells, Bioprocess Engineering*

Joan W. Bennett, Rutgers University, School Environmental and Biological Sciences, New Brunswick, New Jersey, *Aflatoxins*

Erica E. Benson, University of Abertay Dundee, Dundee, Scotland, United Kingdom, *Cryopreservation of Plant Cells Tissues and Organs*

I.S. Bentley, ABM Brewing and Enzymes Group/Rhone-Poulenc, Stockport, United Kingdom, *Enzymes, Starch Conversion*

Claudia Berdugo, The Ohio State University, Columbus, Ohio, *Aeration, Mixing, and Hydrodynamics, Animal Cell Bioreactors*

Sonja Berensmeier, Technische Universität München, Institute of Biochemical Engineering, Garching, Germany, *Bioseparation, Magnetic Particle Adsorbents*

Magnus Bergkvist, College of Nanoscale Science and Engineering (University at Albany), Albany, New York, *Bionanofabrication of Nanostructures and Functional Materials*

Martin Bertau, TU Bergakademie Freiberg, Freiberg, Germany, *Polysiloxanes, Biocatalytic Functionalization*

Joseph Bertolini, CSL Bioplasma, Broadmeadows, Victoria, Australia, *Protein Chromatography, Manufacturing Scale*

Lorena Betancor, Instituto de Catalisis, CSIC, Madrid, Spain, *Immobilized Enzymes*

Michael J. Betenbaugh, Johns Hopkins University, Baltimore, Maryland, *Insect Cells and Larvae, Gene Expression Systems*

Arun K. Bhunia, Molecular Food Microbiology Laboratory, Prude University, West Lafayatte, Indiana, *Biosensors, Foodborne Pathogen Detection*

Rebekka Biedendieck, Protein Science Group, University of Kent, Canterbury, Kent, United Kingdom, *Bacillus Megaterium and Other Bacilli: Industrial Applications*

Horst Bierau, Merck Serono, Protein Chemistry—Biophysical Characterization & PAT, Guidonia (Roma), Italy, *Spectral Data Comparability–Acceptance Criteria Definition by Statistical Analysis*

Alessandra Bini, Università degli Studi di Firenze, Firenze, Italy; Cranfield Health, Cranfield University, Bedfordshire, United Kingdom, *Biosensors, Aptamers (Aptasensors)*

John R. Birch, Lonza Biologics plc, Berkshire, United Kingdom, *Cell Products, Antibodies; Suspension Culture, Animal Cells*

Darcy Birse, Fast Trak Biopharma Services, GE Healthcare, Piscataway, New Jersey, *Cleaning and Sanitization in Downstream Processing*

Dina Biscotti, University of California, California, *Agricultural Biotechnology and Socioeconomic Effects*

Thomas Bley, Dresden University of Technology, Institute of Food Technology and Bioprocess Engineering, Dresden, Germany, *Flow Cytometry*

Janna K. Blum, School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia; Parker H. Petit Institute of Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, Georgia, *Biocatalytic Synthesis of β-lactam Antibiotics*

Andreas S. Bommarius, School of Chemical and Biomolecular Engineering, Georgia Institute of

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Technology, Atlanta, Georgia; Parker H. Petit Institute of Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, Georgia; School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia, *Biocatalytic Synthesis of β-lactam Antibiotics; Enzymes, Enolate Reductases "Old Yellow Enzyme"*

Jan M. Bonga, Natural Resources Canada, Canadian Forest Service—Atlantic Forestry Centre, Fredericton, Canada, *Conifers, Culture and Genetic Engineering*

Sonja Borchert, University of Erlangen-Nuremberg, Henkestr, Erlangen, Germany; Institute of Molecular Enzyme Technology at the Heinrich-Heine-University of Düsseldorf, Research Centre Jülich, Stetternicher Forst, Jülich, Germany, *Enzyme-Catalyzed Asymmetric Reduction of Ketones*

Uwe T. Bornscheuer, University of Greifswald, Institute of Biochemistry, Greifswald, Germany, *Lipases, Synthesis of Chiral Compounds, Aqueous and Organic Solvents; Monooxygenases, Baeyer-Villiger Applications in Organic Synthesis*

Birthe Borup, Codexis Laboratories Singapore Pte Ltd, Analytical Biochemistry, Singapore, *Chiral Alcohols by Enzymatic Preparation*

Richard Bott, Genencor, A Danisco Division, Palo Alto, California, *Proteolytic Cleavage, Reaction Mechanisms*

Eva Branda, University of Perugia, Perugia, Italy, *Basidiomycetous Yeasts for Production of Carotenoids*

Bugarski Branko, University of Belgrade, Belgrade, Republic of Serbia, *Immobilized Cells*

Jan Brazolot, University of Guelph, Guelph, Ontario, Canada, *Toxin Resistant Plants from Plant Cell Culture and Transformation*

Eggert Brekkan, GE Healthcare Bio-Sciences AB, Uppsala, Sweden, *High-Throughput Technologies in Bioprocess Development*

Phil J. Bremer, University of Otago, Dunedin, New Zealand, *Clean-in-Place (CIP)*

Richard Brettell, International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, *Monocots, Tissue Culture*

Susan A. Brooks, School of Life Sciences, Oxford Brookes University, Oxford, United Kingdom, *Protein Glycosylation*

Kurt Brorson, Office of Biotech Products, Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, *Quality by Design (QbD), Biopharmaceutical Manufacture; Virus Retentive Filters*

R. Buccholz, Technical University of Berlin, Berlin, Germany, *Microencapsulation*

Klaus Buchholz, Institute for Chemical Engineering, Technical University, Braunschweig and Institut für Organische Chemie, Universität Würzburg, Am Hubland, Würzburg, Germany, *Enzymatic Oligosaccharide Synthesis; History of Biotechnology*

Boyke Bunk, Institute of Microbiology, Technische Universität Braunschweig, Braunschweig, Germany, *Bacillus Megaterium and Other Bacilli: Industrial Applications*

Jeffrey Burkhardt, University of Florida, Gainesville, Florida, *Agricultural Biotechnology, Social Ethics, and Family Farms*

Thierry Burnouf, Human Protein Process Sciences, Lille, France, *Affinity Chromatography—Fractionated and DNA-Engineered Plasma Proteins*

Michael Butler, University of Manitoba, Winnipeg, Manitoba, Canada, *Energy Metabolism of Cells Used for Industrial Production; Protein Glycosylation, Methods for Determination*

Roxana Butoi, Merck Research Laboratories, Pennsylvania, *Proteins, Thermal Unfolding*

Pietro Buzzini, University of Perugia, Perugia, Italy, *Basidiomycetous Yeasts for Production of Carotenoids*

Joaquim M.S. Cabral, IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal, *Steroid Bioconversion*

Marco A. Cacciuttolo, Percivia LLC, Cambridge, Massachusetts, *Gene Expression, Human Cells*

F. Garcia Camacho, Universidad de Almeria, Almeria, Spain, *Bioreactors, Airlift Reactors*

J.-L. Flores Candia, GBF-Gesellschaft für Biotechnologische Forschung GmbH, Braunschweig, Germany, *Xanthan Gum*

A. Carloni, Cranfield Health, Cranfield University, Cranfield, Bedfordshire, United Kingdom, *Bioprocess Monitoring*

Magnus Carlquist, Lund University, Lund, Sweden; Center for Microbial Biotechnology, Technical University of Denmark, kgs, Lyngby, Denmark, *Bioreduction*

Graham Carpenter, Vanderbilt University School of Medicine, Nashville, Tennessee, *Cell-Surface Receptors: Structure, Activation, and Signaling*

Trent Carrier, Invitrogen, part of Life Technologies, Grand Island, New York, *High-Throughput Technologies in Bioprocess Development*

Alan C. Cassells, National University of Ireland Cork, Ireland, *Contamination Detection and Elimination in Plant Cell Culture*

Artur Cavaco-Paulo, Universidade do Minho, Braga, Portugal, *Biotransformation of Synthetic Fibers*

Jeffrey J. Chalmers, The Ohio State University, Columbus, Ohio, *Aeration, Mixing, and Hydrodynamics, Animal Cell Bioreactors*

Leslie Chan, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia, *Baculovirus Kinetics in Insect Cell Culture*

Perng-Kuang Chang, USDA/ARS, Southern Regional Research Center, New Orleans, Louisiana, *Aflatoxins*

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Timothy S. Charlebois, Genetics Institute, Andover, Massachusetts, *Cell Stability, Animal*

Marvin Charles, Lehigh University, Bethlehem, Pennsylvania, *Fermenter Design*

Peter S.J. Cheetham, Zylepsis, Ltd., Ashford, United Kingdom, *Enzymes for Flavor Production*

Aaron Chen, Oceanside Process Research & Development, Genentech. Inc, Oceanside, California, *Animal Cell Culture Media*

Guo-Qiang Chen, Tsinghua University, Beijing, China; Multidisciplinary Research Center, Shantou University, Guangdong, China, *Polyhydroxyalkanoates (PHAs): Separation, Purification and Manufacturing Methods*

Sébastien Chenuet, Institute of Bioengineering, Laboratory of Cellular Biotechnology, École Polytechnique Fédéral de Lausanne (EPFL), Lausanne, Switzerland, *Chinese Hamster Ovary Cells, Recombinant Protein Production*

Yusuf Chisti, School of Engineering, Massey University, Palmerston North, New Zealand, *Mass Transfer; Shear Sensitivity; Solid Substrate Fermentations, Enzyme Production, Food Enrichment*

John Chon, Percivia LLC, Cambridge, Massachusetts, *Gene Expression, Human Cells*

Douglas B. Chrisey, Rensselaer Polytechnic Institute, Troy, New York, *Bioprinting*

Jens Christensen, Merck & Co. Inc, Rahway, New Jersey, *Vent Gas Analysis*

David Clark, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

Harald Claus, Institut für Mikrobiologie und Weinforschung, Johannes Gutenberg-Universität Mainz, Mainz, Germany, *Laccase*

Tim Clayton, Glaxo Wellcome, Beckenham, Kent, United Kingdom, *Immunoregulators; Viral Gene Therapy Vectors*

Thomas E. Cleveland, USDA/ARS, Southern Regional Research Center, New Orleans, Louisiana, *Aflatoxins*

James A. Coker, University of Maryland, Baltimore, Maryland, *Halophiles, Industrial Applications*

Steve Collier, Codexis Laboratories Singapore Pte Ltd, Chemical Development, Singapore, *Chiral Alcohols by Enzymatic Preparation*

Attilio Converti, University of Genoa, Genoa, Italy, *Biofilters for Air Purification*

Thibaud Coradin, UPMC Univ Paris 6, CNRS and Collège de France, Chimie de la Matière Condensée de Paris, Paris, France, *Biocers, Industrial Applications*

Athel Cornish-Bowden, Unité de Bioénergétique et Ingénierie des Protéines, Centre National de la Recherche Scientifique, Marseilles, France, *Kinetics, Enzymes*

David T. Corr, Rensselaer Polytechnic Institute, Troy, New York, *Bioprinting*

Rosalie J. Cote, Becton Dickinson Microbiology Systems, Sparks, Maryland, *Media Composition, Microbial, Laboratory Scale*

Deborah Court, University of Manitoba, Winnipeg, Manitoba, Canada, *Energy Metabolism of Cells Used for Industrial Production*

David J. Craik, The University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia, *Cyclotides*

Jorge H. Crosa, Oregon Health and Sciences University, Portland, Oregon, *Plasmid DNA Replication*

Efrem Curcio, University of Calabria, Arcavacata di Rende (CS), Italy, *Protein Crystallization, Kinetics*

Wayne R. Curtis, The Pennsylvania State University, University Park, Pennsylvania, *Hairy Roots, Bioreactor Growth*

Alessandro D'Aprano, University of Rome, La Sapienza, Rome, Italy, *Conductivity*

Hans Von Döhren, Technical University Berlin, Institute of Chemistry, Berlin, Germany, *Peptides*

Heike Dörnenburg, University of Erlangen-Nuremberg, Institute of Bioprocess Engineering, Erlangen, Germany, *Cyclotides*

Stefan Dübel, Technische Universität Braunschweig, Institute of Biochemistry and Biotechnology, Spielmannstr, Braunschweig, Germany, *Antibody Production: Human, Recombinant*

Peter Dürre, Institut für Mikrobiologie und Biotechnologie, Universität Ulm, Ulm, Germany, *Clostridium*

Paul A. Dalby, University College London, London, United Kingdom, *Transketolases*

Tracy R. Daniels, University of California at Los Angeles, Los Angeles, California, *Monoclonal Antibodies, Human, Engineered*

Priya Dassarma, University of Maryland, Baltimore, Maryland, *Halophiles, Industrial Applications*

Shiladitya Dassarma, University of Maryland, Baltimore, Maryland, *Halophiles, Industrial Applications*

Thomas Daussmann, Codexis Laboratories Singapore Pte Ltd, Chemical Development, Singapore, *Chiral Alcohols by Enzymatic Preparation*

M.R. Davey, University of Nottingham, School of Biosciences, Sutton Bonington Campus, Loughborough, United Kingdom, *Plant Protoplasts*

John M. Davis, School of Life Sciences, University of Hertfordshire, Hatfield, Hertfordshire, United Kingdom, *Aseptic Techniques in Cell Culture*

Andria L. Deaguero, School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia; Parker H. Petit Institute of Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, Georgia, *Biocatalytic Synthesis of β-lactam Antibiotics*

W.-D. Deckwer, GBF-Gesellschaft für Biotechnologische Forschung GmbH, Braunschweig, Germany, *Xanthan Gum*

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Frank Delvigne, Fond de la Recherche Scientifique (FRNS-FRS), Bruxelles, Belgium; Faculté Universitaire des Sciences Agronomiques, Unité de Bio-industries/CWBI, Gembloux, Belgium, *Foam Formation and Control in Bioreactors*

Arnold L. Demain, Charles A. Dana Research Institute for Scientists Emeriti (RISE), Drew University, Madison, New Jersey, *Biosynthesis of Microbial Primary Metabolites*

Brian Derby, School of Materials, Materials Science Centre, University of Manchester, United Kingdom, *Bioprinting, Inkjet Deposition*

Jean Didelez, SmithKline Beecham Biologicals, Rixensart, Belgium, *Current Good Manufacturing Practice (cGMP) Compliance for Production Rooms*

C.P. Dillon, C.P. Dillon & Associates, Hurricane, West Virginia, *Stainless Steels*

Dennis Dobie, Fluor Daniel, Marlton, New Jersey, *Heating, Ventilation, and Air Conditioning*

Ed Domanico, Tri-Clover, Valencia, California, *Pumps, Industrial*

Pauline M. Doran, Monash University, Australia, *Bioreactors, Stirred Tank for Plant Cells Culture*

Barbara Doyle-Prestwich, National University of Ireland Cork, Ireland, *Contamination Detection and Elimination in Plant Cell Culture*

Denis Drapeau, Genetics Institute, Andover, Massachusetts, *Cell Stability, Animal*

Hans G. Drexler, DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, *Mycoplasma Contamination of Cell Cultures*

Enrico Drioli, Institute on Membrane Technology, ITM-CNR, At University of Calabria, Rende, Italy, *Biocatalytic Membrane Reactors; Protein Crystallization, Kinetics*

Jason Duex, University of Colorado Denver, Aurora, Colorado, *Growth Factors: Protein Processing, Endocytosis, and Intracellular Sorting*

Dominique J. Dumet, University of Abertay Dundee, Dundee, Scotland, United Kingdom, *Cryopreservation of Plant Cells, Tissues and Organs*

Eva Maria Egelseer, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

Lothar Eggeling, Institute of Biotechnology, Juelich, Germany, *Microbial Metabolite Export*

Dieter Eibl, Zurich University of Applied Sciences, School of Life Sciences and Facility Management, Institute of Biotechnology, Wädenswil, Switzerland, *Antibody Manufacture, Disposable Systems; Culture Establishment, Plant Cell*

Regine Eibl, Zurich University of Applied Sciences, School of Life Sciences and Facility Management, Institute of Biotechnology, Wädenswil, Switzerland, *Antibody Manufacture, Disposable Systems; Culture Establishment, Plant Cell*

Lynda B.M. Ellis, Minneapolis, Minnesota, *Database Tools for Microbial Biocatalysis*

Takakazu Endo, Nitto Chemical Industry Co., Ltd., Tokyo, Japan, *Nitrile Hydratase*

Larry E. Erickson, Kansas State University, Manhattan, Kansas, *Anaerobes*

Bruno Fabiano, G.B. Bonino, University of Genoa, Genoa, Italy, *Microbiological Induced Corrosion and Inhibition*

Zhiwu Fang, Amgen Inc., Systems Informatics, Thousand Oaks, California, *Large-Scale Chromatography Columns, Modeling Flow Distribution*

Pierre Fauquembergue, Gist-brocades, Seclin Cedex, France, *Enzymes, Fruit Juice Processing*

Gao Fei, National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China, *Enzyme Immobilization, Biocatalyst Featured with Nanoscale Structure*

J. Ricardo Fernández-Fernández, Chemical and Bioprocess Engineering Department, Pontificia Universidad Católica de Chile, Santiago, Chile, *Solid Substrate Fermentation, Automation*

Mario Fernández-Fernández, Universidad de Talca, Talca, Región del Maule, Chile, *Solid Substrate Fermentation, Automation*

Pedro Fernandes, IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal, *Steroid Bioconversion*

Gloria Fernandez-Lorente, Instituto de Catalisis, CSIC, Madrid, Spain, *Immobilized Enzymes*

James T. Fleming, University of Tennessee, Center for Environmental Biotechnology, Knoxville, Tennessee, *Bioelectronics*

Patrick Florent, SmithKline Beecham Biologicals, Rixensart, Belgium, *Current Good Manufacturing Practice (cGMP) Compliance for Production Rooms*

Ian G. Fotheringham, NSC Technologies, Mount Prospect, Illinois, *Phenylalanine*

Mark Richard Fowler, Leicester School of Pharmacy, De Montfort University, Leicester, United Kingdom, *Cell Cycle in Suspension Cultured Plant Cells; Plant Cell Culture, Laboratory Techniques*

Peter Fröhlich, TU Bergakademie Freiberg, Freiberg, Germany, *Polysiloxanes, Biocatalytic Functionalization*

Matthias Franzreb, Karlsruhe Institute of Technology, Institute for Functional Interfaces, Eggenstein-Leopoldshafen, Germany, *Bioseparation, Magnetic Particle Adsorbents*

Juan Luis De La Fuente, R&D Biology, Antibióticos S. A., León, Spain, *Cephalosporin Production by Fungal Metabolic Engineering*

Daniel Y. C. Fung, Kansas State University, Manhattan, Kansas, *Anaerobes*

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Renata L.A. Furlan, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil, *Anticancer Compounds Development, Structural Modifications and Therapeutic Applications*

Pete Gagnon, Validated Biosystems, San Clemente, California, *Chromatographic Purification of Virus Particles*

Sean R. Gallagher, Motorola Phoenix Corporate Research Laboratories, Tempe, Arizona, *Electrophoresis of Proteins and Nucleic Acids*

Mallikarjunarao Ganesana, Clarkson University, Potsdam, New York, *Biosensors, Toxicity Monitoring*

F.A.P. Garcia, University of Coimbra, Coimbra, Portugal, *Cell Wall Disruption*

Felix Garcia-Ochoa, Facultad Quimicas, Universidad Complutense, Madrid, Spain, *Oxygen Transfer Rate Determination: Chemical, Physical and Biological Methods*

Maria Gavrilescu, Research Centre for Antibiotics, Iaşi, Romania, *Static Mixing in Fermentation Processes*

Martin Gawlitzek, Late Stage Cell Culture, Genentech, Inc., San Francisco, California, *Animal Cell Culture Media*

Craig J.L. Gershater, S.B. Pharmaceuticals, Harlow, Essex, England, *Inoculum Preparation*

Tom Gervais, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

Iraj Ghazi, The Ohio State University, Columbus, Ohio, *Large-Scale Protein Purification, Self-Cleaving Aggregation Tags*

Haile Ghebremariam, Medarex, Inc., Bloomsbury, New Jersey, *Inoculum Expansion Methods, Recombinant Mammalian Cell Lines*

Robert L. Gherna, American Type Culture Collection, Rockville, Maryland, *Culture Preservation*

Maria L. Ghirardi, National Renewable Energy Laboratory, Golden, Colorado, *Algal Hydrogen Production*

Oreste Ghisalba, Ghisalba Life Sciences, Reinach, Switzerland, *Industrial Biotransformation*

Siddartha Ghose, Aston University, Birmingham, United Kingdom, *Protein Adsorption, Expanded Bed*

Carlo E. Giartosio, Merck Serono, Protein Chemistry—Biophysical Characterization & PAT, Guidonia (Roma), Italy, *Spectral Data Comparability–Acceptance Criteria Definition by Statistical Analysis*

Friedrich Giffhorn, Applied Microbiology, Saarland University, Saarbruecken, Germany, *Dehydrogenases, Electrochemical Cofactor Regeneration*

Assunta Giordano, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Napoli, Italy, *Enzymatic Transglycosylation*

Lidietta Giorno, Institute on Membrane Technology, ITM-CNR, At University of Calabria, Rende, Italy, *Biocatalytic Membrane Reactors*

Paolo Giudici, University of Modena and Reggio Emilia, Reggio Emilia, Italy, *Acetic Acid Bacteria, Biotechnological Applications*

Leland Glenna, Pennsylvania State University, Pennsylvania, *Agricultural Biotechnology and Socioeconomic Effects*

Guy Godeau, SmithKline Beecham Biologicals, Rixensart, Belgium, *Current Good Manufacturing Practice (cGMP) Compliance for Production Rooms*

Ruben Godoy-Silva, The Ohio State University, Columbus, Ohio; Universidad Nacional de Colombia, Bogotá, Colombia, *Aeration, Mixing, and Hydrodynamics, Animal Cell Bioreactors*

Emilio Gomez, Facultad Quimicas, Universidad Complutense, Madrid, Spain, *Oxygen Transfer Rate Determination: Chemical, Physical and Biological Methods*

Marta Goretti, University of Perugia, Perugia, Italy, *Basidiomycetous Yeasts for Production of Carotenoids*

Johnathan Gorke, BioTechnology Institute, University of Minnesota, Minnesota, *Enzyme-Catalyzed Reactions in Ionic Liquids*

Marie-F Gorwa-Grauslund, Lund University, Lund, Sweden, *Bioreduction*

Susanne Gräslund, Structural Genomics Consortium, Karolinska Institutet, Stockholm, Sweden, *Affinity Fusions, Gene Expression*

Harald Gröger, University of Erlangen-Nuremberg, Henkestr, Germany, *Enzyme-Catalyzed Asymmetric Reduction of Ketones*

K. Grage, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications*

Catherine Grassin, Gist-brocades, Seclin Cedex, France, *Enzymes, Fruit Juice Processing*

David R. Gray, Chiron Corporation, Emeryville, California, *Bioreactor Operations*

Steven R. Gray, North Carolina State University, Raleigh, North Carolina, *Microorganisms, Extremely Thermophilic*

Thomas Graycar, Genencor, A Danisco Division, Palo Alto, California, *Proteolytic Cleavage, Reaction Mechanisms*

J. Bryan Griffiths, Scientific Consultancy & Publishing, Salisbury, United Kingdom, *Mammalian Cell Culture Reactors, Scale-Up*

Emilio Molina Grima, University of Almería, Almería, Spain, *Microalgae, Mass Culture Methods*

M. Michael Gromiha, Computational Biology Research Center (CBRC), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan, *Temperature-Dependent Molecular Adaptations, Microbial Proteins*

Tingyue Gu, Ohio University, Athens, Ohio, *Chromatography, Radial Flow*

S. Guillouet, Massachusetts Institute of Technology, Cambridge, Massachusetts, *Corynebacteria, Brevibacteria*

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Jose M. Guisan, Instituto de Catalisis, CSIC, Madrid, Spain, *Immobilized Enzymes*

Maria Gullo, University of Modena and Reggio Emilia, Reggio Emilia, Italy, *Acetic Acid Bacteria, Biotechnological Applications*

David L. Hacker, Institute of Bioengineering, Laboratory of Cellular Biotechnology, École Polytechnique Fédéral de Lausanne (EPFL), Lausanne, Switzerland, *Chinese Hamster Ovary Cells, Recombinant Protein Production; Transient Gene Expression in Mammalian Cells*

Mélanie Hall, School of Chemical and Biomolecular Engineering; Parker H. Petit Institute of Bioengineering and Biosciences, *Enzymes, Enolate Reductases "Old Yellow Enzyme"*

Martin Hammarström, Structural Genomics Consortium, Karolinska Institutet, Stockholm, Sweden, *Affinity Fusions, Gene Expression*

William Hannah, Michigan State University—Philosophy, Michigan, *Agricultural Biotechnology, Food Safety, Risk, Consent, and Ethics*

Elo Harald Hansen, University of Denmark Lyngby, Denmark, *Flow Injection Analysis (FIA)*

Michael A. Hanson, Bio-Manufacturing Sciences Group, Pfizer, New York, *Biominiaturization of Bioreactors*

Satoshi Hanzawa, TOSOH Corp., Ayase-shi and Tokyo Research Laboratory, Hayakawa, Japan, *Aspartame; Thermolysin*

Colin Harbour, The University of Sydney, NSW, Australia, *Contamination Detection in Animal Cell Culture*

Keith Harding, Scottish Crop Research Institute, Dundee, Scotland, United Kingdom, *Cryopreservation of Plant Cells Tissues and Organs*

James M. Harris, North Carolina State University, Raleigh, North Carolina, *Enzymes, Extremely Thermophilic*

Scott Harrison, Genetics Institute, Andover, Massachusetts, *Cell Stability, Animal*

Martin Hartmann, University of Augsburg, Augsburg, Germany, *Immobilization of Proteins and Enzymes, Mesoporous Supports*

Richard Hassett, Invitrogen, part of Life Technologies, Grand Island, New York, *High-Throughput Technologies in Bioprocess Development*

Rudolf Hausmann, Institut für Bio-und Lebensmitteltechnik, Bereich II: Technische Biologie, Universität Karlsruhe (TH), Engler-Bunte-Ring 1, Karlsruhe, Germany, *Biosurfactants, Rhaminolipid, Microbial Production*

Martin Hedström, Lund University, Lund, Sweden, *Sampling and Sample Handling for Process Control*

Joseph J. Heijnen, Delft University of Technology, Delft, The Netherlands, *Bioenergetics of Microbial Growth*

K. Heilmann, University of Potsdam, Institute of Biochemistry and Biology, Potsdam, Germany, *Animal Cell Types, Hybridoma Cells*

Eva Heldin, GE Healthcare Bio-Sciences AB, Uppsala, Sweden, *High-Throughput Technologies in Bioprocess Development*

Gustavo Helguera, University of California at Los Angeles, Los Angeles, California, *Monoclonal Antibodies, Human, Engineered*

María J. Hernáiz, Servicio de Interacciones Moleculares, Parque Científico de Madrid, Facultad de Farmacia, Universidad Complutense, Madrid, Spain, *Enzyme-Catalyzed Synthesis of Nonnatural or Modified Nucleosides*

Birgit Hickstein, Clausthal University of Technology, Institute of Chemical Process Engineering, Clausthal-Zellerfeld, Germany, *Bioseparation, Magnetic Particle Adsorbents*

Lars Hillringhaus, Institut für Organische Chemie, Universität Würzburg, Am Hubland, Würzburg, Germany, *Glycochips*

Lutz Hilterhaus, Institute of Technical Biocatalysis, Hamburg University of Technology, Hamburg, Germany, *Biotransformation, Process Optimization, Kinetics and Engineering Aspects*

E. Dan Hirleman, School of Mechanical Engineering, Purdue University, West Lafayette, Indiana, *Biosensors, Foodborne Pathogen Detection*

Yamamoto Hiroaki, Corporate R&D Center, R&D Management, Daicel Chemical Industries, Ltd, Himeji-shi, Hyogo, Japan, *Carbonyl Reductase*

Imanaka Hiroyuki, Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan, *Membrane-Surface Liquid Culture, Fungi*

Richard B. Hitchman, Oxford Expression Technologies Ltd., Oxford Brookes University, United Kingdom, *Baculovirus Expression Systems*

Bernd Hitzmann, Institut für Technische Chemie, Gottfried Wilhelm Leibniz Universität Hannover, Hannover, Germany, *Fluorescence Techniques for Bioprocess Monitoring*

Timothy John Hobley, Technical University of Denmark, Systems of Biology, Lyngby, Denmark, *Bioseparation, Magnetic Particle Adsorbents*

Paul Holmes, University College Dublin, Belfield, Dublin, Ireland, *Cell Cycle Synchronization*

H.J.G. Ten Hoopen, Delft University for Technology, Delft, The Netherlands, *Bioreactors, Continuous Culture of Plant Cells; Recirculation Bioreactor in Plant Cell Culture*

Masashi Hosokawa, Faculty of Fisheries Sciences, Hokkaido University, Hokkaido, Japan, *Edible Oils, Microbial Modification Processes*

Ching T. Hou, Renewable Products Technology Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, Illinois, *Edible Oils, Microbial Modification Processes*

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Wei-Shou Hu, University of Minnesota, Minneapolis, Minnesota, *Mammalian Cell Bioreactors*

Weiwei Hu, Cell Culture Development, Biogen Idec Inc., San Diego, California, *Bioreactors, Cell Culture, Commercial Production*

Erwin Huebner, University of Manitoba, Winnipeg, Manitoba, Canada, *Characterization of Cells, Microscopic*

Holger Huebner, Technical University of Berlin, Berlin, Germany, *Microencapsulation*

Werner Hummel, Institute of Molecular Enzyme Technology at the Heinrich-Heine-University of Düsseldorf, Research Centre Jülich, Stetternicher Forst, Jülich, Germany, *Enzyme-Catalyzed Asymmetric Reduction of Ketones*

H. Hummel, Philips Research Laboratories, Aachen, Germany, *Luminescent Materials, Biological Applications*

Tony Hunt, Advanced Minerals Corporation, Santa Barbara, California, *Filter Aids*

Valerie Huse, University of Maryland, Baltimore, Maryland, *Halophiles, Industrial Applications*

Sunyia Hussain, Johns Hopkins University, Baltimore, Maryland, *Insect Cells and Larvae, Gene Expression Systems*

Yasuhiro Ikenaka, Kaneka Corporation, Hyogo, Japan, *Aminohydrolases for Production of* D-*Amino Acids*

Nicola Ilk, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

W.M. Ingledew, University of Saskatchewan, Parksville, Canada; Ethanol Technology Institute, Parksville, Canada, *Ethanol Fuel Production, Yeast Processes*

Masayuki Inui, Research Institute of Innovative Technology for the Earth (RITE), Kizugawa, Kyoto, Japan, *Amino Acids, Branched Chain,* L-*Isoleucine; L-Aspartic Acid, Production Processes*

Hisao Ito, Fermentation and Biotechnology Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan, *Amino Acid Production,* L-*Lysine*

Maria J. Guardia, Camino De Purchill, Puleva Biotech Department of Process Engineering, Granada, Spain, *Mammalian Cell Bioreactors*

Dieter Jahn, Institute of Microbiology, Technische Universität Braunschweig, Braunschweig, Germany, *Bacillus Megaterium and Other Bacilli: Industrial Applications*

A.C. Jahns, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications*

Steen Weber Jensen, Novo Nordisk A/S, Bagsvaerd, Denmark, *Insulin Purification*

Toru Jojima, Research Institute of Innovative Technology for the Earth (RITE), Kizugawa, Kyoto, Japan, *Amino Acids, Branched Chain,* L-*Isoleucine*

Carl S. Jone, Merck Serono, Protein Chemistry—Biophysical Characterization & PAT, Guidonia (Roma), Italy, *Spectral Data Comparability–Acceptance Criteria Definition by Statistical Analysis*

Jeong Chan Joo, School of Chemical and Biological Engineering, Seoul National University, Seoul, Korea, *Thermostable Proteins*

Omkar Joshi, Bayer HealthCare LLC, Berkeley, California, *Adsorption, Proteins with Synthetic Materials*

T. Juestel, Philips Research Laboratories, Aachen, Germany; University of Applied Sciences Muenster, Steinfurt, Germany, *Luminescent Materials, Biological Applications*

Dirk Jung, Advanced Materials Science, Department of Physics, University of Augsburg, Augsburg, Germany, *Immobilization of Proteins and Enzymes, Mesoporous Supports*

Beth H. Junker, Bioprocess R&D, Merck Research Laboratories, Rahway, New Jersey, *Good Manufacturing Practice (GMP) and Good Industrial Large Scale Practice (GLSP); Pilot Plants, Design and Operation*

Per Kårsnäs, Institute of Biology and Chemical Engineering, Mälardalens högskola, Eskilstuna, Sweden, *Chromatography, Hydrophobic Interaction*

Christian Kaisermayer, Project Manager Cell Culture Applications & Support, GE Healthcare, Vienna, Austria, *Microcarrier Culture*

Michael S. Kallos, Pharmaceutical Production Research Facility (PPRF), Schulich School of Engineering, University of Calgary, Alberta, Canada, *Neural Stem Cells, Bioprocess Engineering*

Manohar Kalyanpur, Consultant, Bioseparations & Pharmaceutical Validation, Plaisir, France, *Membrane Separations*

Ingo Kampen, Technische Universität, Institute for Particle Technology, Braunschweig, Germany, *Cell Disruption, Microbial, Micromechanical Properties*

Shamsher S. Kanwar, H.P. University, Summer Hill, Shimla, *Lipases*

András Kapus, Toronto Hospital, Toronto, Ontario, Canada, *Membrane Structure and Transport of Small Molecules and Ions*

Selin Kara, Institute of Technical Biocatalysis, Hamburg University of Technology, Hamburg, Germany, *Enzymatic C-C Bond Formation, Asymmetric*

Levente Karaffa, Faculty of Science and Technology, University of Debrecen, Debrecen, Hungary, *Citric Acid Processes*

R.S. Karthikeyan, Indian Institute of Technology–Madras, Chennai, India, *Dextran, Microbial Production*

Michihiko Kataoka, Kyoto University, Kyoto, Japan, *Aldehyde Reductase; Lactonohydrolase; Pantothenic Acid and Related Compounds*

Takane, Katayama, Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University,

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Japan, *L-DOPA, Microbial Production; Tyrosine Phenol-lyase*

Yasuo Kato, Toyama Prefectural University, Toyama, Japan, *Opine Dehydrogenase, Secondary Amine Dicarboxylic Acids*

Randal J. Kaufman, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan, *Protein Synthesis and Secretion, Animal Cells*

Takuo Kawamoto, Kyoto University, Kyoto, Japan, *Organosilicon Compounds, Enzymatic Modification*

Romas Kazlauskas, University of Minnesota, Minnesota; BioTechnology Institute, University of Minnesota, Minnesota, *Enzyme-Catalyzed Reactions in Ionic Liquids*

Nakanishi Kazuhiro, Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan, *Membrane-Surface Liquid Culture, Fungi*

Robert M. Kelly, North Carolina State University, Raleigh, North Carolina, *Enzymes, Extremely Thermophilic; Microorganisms, Extremely Thermophilic*

Christian Kennes, Chemical Engineering Laboratory, Faculty of Sciences, University of La Coruña, La Coruña, Spain, *Biofilters for Air Purification*

Mansoor A. Khan, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, United States Food and Drug Administration, *Quality by Design (QbD), Biopharmaceutical Manufacture*

Soo Hean Gary Khoo, University College Dublin, School of Chemical and Bioprocess Engineering, Belfield, Ireland, *Apoptosis*

Shun-ICHI Kidokoro, Sagami Chemical Research Center, Kanagawa, Japan, *Thermolysin*

Linda A. King, School of Life Sciences, Oxford Brookes University, United Kingdom, *Baculovirus Expression Systems*

Shukuo Kinushita, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, *L-Glutamic Acid Production*

Shigenobu Kishino, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, *Oils, Microbial Production*

Robert Kiss, Late Stage Cell Culture, Genentech, Inc., San Francisco, California, *Animal Cell Culture Media*

Robbert Kleerebezem, Delft University of Technology, Delft, The Netherlands, *Bioenergetics of Microbial Growth*

Geert-Jan De Klerk, Wageningen Tissue Culture Center, BU Biodiversity and Breeding, AA Wageningen, The Netherlands, *Adventitious Organogenesis*

Krystyna Klimaszewska, Natural Resources Canada, Canadian Forest Service—Laurentian Forestry Centre, Quebec, Canada, *Conifers, Culture and Genetic Engineering*

Claudia Kloth, Medarex, Inc., Bloomsbury, New Jersey, *Inoculum Expansion Methods, Recombinant Mammalian Cell Lines*

George K. Knopf, The University of Western Ontario, Mechanical and Materials Engineering, London, Ontario, Canada, *Biophotoreceptor Arrays*

Arthur L. Koch, Indiana University, Bloomington, Indiana, *Microbial Growth Measurement*

Gert-Wieland Kohring, Applied Microbiology, Saarland University, Saarbruecken, Germany, *Dehydrogenases, Electrochemical Cofactor Regeneration*

Fragiskos N. Kolisis, School of Chemical Engineering, National Technical University of Athens, Athens, Greece, *Reverse Micelles, Enzymes*

Hidenobu Komeda, Toyama Prefectural University, Toyama, Japan, *D-Aminopeptidase and Alkaline D-Peptidase*

Dhinakar S. Kompala, University of Colorado, Boulder, Colorado, *Cell Growth and Protein Expression Kinetics*

Jone Koo, Korea Advanced Institute of Science and Technology, Daejon, Korea, *Osmolarity Effects, Chinese Hamster Ovary Cell Culture*

Petra Kornberger, Applied Microbiology, Saarland University, Saarbruecken, Germany, *Dehydrogenases, Electrochemical Cofactor Regeneration*

Sergey Kosourov, National Renewable Energy Laboratory, Golden, Colorado, *Algal Hydrogen Production*

Alexandros Koulouris, Intelligen Europe, Thermi, Greece, *Bioprocess Design, Computer-Aided*

Udo Kragl, Rostock University, Rostock, Germany, *Biotransformation, Process Optimization, Kinetics and Engineering Aspects*

Marina Kraußer, University of Erlangen-Nuremberg, Henkestr, Erlangen, Germany, *Enzyme-Catalyzed Asymmetric Reduction of Ketones*

Christian P. Kubicek, Vienna University of Technology, Institute of Chemical Engineering, Vienna, Austria, *Citric Acid Processes*

Maria-Regina Kula, Heinrich Heine University Düsseldorf, Jülich, Germany, *Protein Purification, Aqueous Liquid Extraction*

Hidehiko Kumagai, Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Japan, *Tyrosine Phenol-lyase; L-DOPA, Microbial Production*

Sandeep Kumar, Pharmaceutical Research and Development, Global Biologics, Pfizer Global Research and Development, Chesterfield, Missouri, *Temperature-Dependent Molecular Adaptations, Microbial Proteins*

Arno Kwade, Technische Universität, Institute for Particle Technology, Braunschweig, Germany, *Cell Disruption, Microbial, Micromechanical Properties*

Andreas Lübbert, Institute of Biotechnology; Centre of Bioengineering, Martin-Luther-University Halle-Wittenberg, Weinbergweg, Halle (Saale), Germany, *Gas Holdup in Bioreactors*

William B. Lacy, University of California, Davis, California, *Agricultural Biotechnology and Socioeconomic Effects*

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Marcelo Fernández Lahore, Downstream Bioprocessing Laboratory, School of Engineering and Science, Jacobs University, Bremen, Germany, *Expanded Bed Chromatography, Surface Energetics of Biomass Deposition*

Philippe Lam, Pharmaceutical Development Genentech, Inc., South San Francisco, California, *Freezing, Biopharmaceutical Products*

Philippe Langella, Unité d'Ecologie et Physiologie du Système Digestif (UR910), INRA, Jouy-en-Josas, France, *Secretion of Heterologous Proteins, Gram-Positive Bacteria, Lactococcus lactis*

David Laporte, University of Minnesota, Minneapolis, *Glyoxylate Bypass of Escherichia Coli, Regulation*

Alvaro R. Lara, Universidad Autónoma Metropolitana-Cuajimalpa, *Bioreactor Scale-Down*

Per Larsen, Novo Nordisk A/S, Bagsvaerd, Denmark, *Insulin Purification*

Chung Lim Law, The University of Nottingham, Malaysia Campus, Selangor, Malaysia, *Drying of Biological Materials*

David J. Leak, College London, London, *Methylotrophs, Industrial Applications*

Jean-Paul Lecomte, Dow Corning S.A., Seneffe, Belgium, *Foam Formation and Control in Bioreactors*

Anh LeDuy, Laval University, Québec, Canada, *Pullulan, Microbial Production Methods*

Pyung Cheon Lee, Ajou University, Suwon, South Korea, *Carotenoids, Microbial Processes*

Gene Lee, Percivia LLC, Cambridge, Massachusetts, *Gene Expression, Human Cells*

Gyun Min Lee, Korea Advanced Institute of Science and Technology, Daejon, Korea, *Osmolarity Effects, Chinese Hamster Ovary Cell Culture*

Lasse Lensu, Lappeenranta University of Technology, Lappeenranta, Finland, *Biosensors, Color-Sensitive*

Mark Leonard, Genetics Institute, Andover, Massachusetts, *Cell Stability, Animal*

P.A. Lessard, Massachusetts Institute of Technology, Cambridge, Massachusetts, *Corynebacteria, Brevibacteria*

John Lewis, Crucell NV, Leiden, The Netherlands, *Gene Expression, Human Cells*

Feng Li, Oceanside Process Research & Development, Genentech. Inc, Oceanside, California, *Animal Cell Culture Media*

Jincai Li, Oceanside Process Research & Development, Genentech. Inc, Oceanside, California, *Animal Cell Culture Media*

Chen-Zhong Li, Nanobioengineering and Bioelectronics Lab, Florida International University, Miami, Florida, *Nanomaterials Incorporated Bioelectronics*

Andreas Liese, Institute of Technical Biocatalysis, Hamburg University of Technology, Hamburg, Germany, *Biotransformation, Process Optimization, Kinetics and Engineering Aspects; Enzymatic C-C Bond Formation, Asymmetric*

Yun Lin, University of Ottawa, Ottawa, Canada, *Pullulan, Microbial Production Methods*

Carsten Lindemann, Boehringer Ingelheim Pharma GmbH & Co. Biberach/ Riß, Germany, *Fluorescence Techniques for Bioprocess Monitoring*

De-Hua Liu, Tsinghua University, Beijing, China, *1,2 and 1,3 Propanediol, Microbial Production Methods*

Jinsong Liu, Product Development, Abraxis BioScience, Melrose Park, Illinois, *Freeze-Drying, Pharmaceuticals*

Jacques Livage, UPMC Univ Paris 6, CNRS and Collège de France, Chimie de la Matière Condensée de Paris, Paris, France, *Biocers, Industrial Applications*

Beth Loberant, Desert Labs, Kibbutz Yotvata, Israel, *Micropropagation of Plants*

K.C. Lowe, University of Nottingham, School of Life and Environmental Sciences, University Park, Nottingham, United Kingdom, *Plant Protoplasts*

Scott Lute, Office of Biotech Products, Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, *Virus Retentive Filters*

Susann Müller, UFZ, Helmholtz Centre for Environmental Research, Department of Environmental Microbiology, Leipzig, Germany, *Flow Cytometry*

Suzanne Ma, University of California Los Angeles, Los Angeles, California, *Antibiotics, Polyketide*

Guang-Hui Ma, National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China, *Enzyme Immobilization, Biocatalyst Featured with Nanoscale Structure*

Glenn Maclsaac, Medarex, Inc., Bloomsbury, New Jersey, *Inoculum Expansion Methods, Recombinant Mammalian Cell Lines*

Pérola O. Magalhães, University of Brasília, Brasília, DF, Brazil, *Lipopolysaccharide, LPS removal, Depyrogenation*

Melissa J. Mahoney, Cornell University, Ithaca, New York, *Extracellular Matrix and Cell Adhesion*

Robert Z. Maigetter, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

Pinching Maness, National Renewable Energy Laboratory, Golden, Colorado, *Algal Hydrogen Production*

J.C. Marcos, Center of Chemistry, University of Minho, Campus de Gualtar, Braga, Portugal, *Plasmid Purification, Therapeutic Applications*

Sonny S. Mark, University of Pennsylvania Medical Center, Pensylvania, *Bionanofabrication of Nanostructures and Functional Materials*

Julia F. Markusen, Bioprocess Research & Development, Merck Research Laboratories, Rahway, NJ, *Monoclonal Antibody Production, Cell Lines*

Robert E. Marquis, University of Rochester Medical Center, Rochester, New York, *Solute Transport, Microbial*

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Dirk E. Martens, Food and Bioprocess Engineering Group, Wageningen University, The Netherlands, *Flux Analysis of Mammalian Cell Culture*

Kenneth A. Marx, Center for Intelligent Biomaterials, Department of Chemistry, University of Massachusetts Lowell, Massachusetts, *Smart Biomaterials and Biosensors: Intelligent Properties of Biomolecules and Cells in Hybrid Devices*

Marco Mascini, Università degli Studi di Firenze, Firenze, Italy, *Biosensors, Aptamers (Aptasensors)*

Bernard Massie, Institut de recherche en biotechnologie, Conseil national de recherches du Canada, Montréal, Québec, Canada, *Transcription, Translation, and the Control of Gene Expression in Mammalian Cells*

Seiji Masuda, Kyoto University, Kyoto, Japan, *Erythropoietin*

Teresa MatamÁ, Universidade do Minho, Braga, Portugal, *Biotransformation of Synthetic Fibers*

Esha Mathew, Cornell University, Ithaca, New York, *Bionanofabrication of Nanostructures and Functional Materials*

Hiroaki Matsumae, Tanabe Seiyaku Co., Ltd., Osaka, Japan, *Diltiazem Synthesis*

Bo Mattiasson, Lund University, Lund, Sweden, *Sampling and Sample Handling for Process Control*

Karl-Heinz Maurer, Henkel AG & Co., KGaA, Düsseldorf, *Enzymes, Detergent*

Joseph McGuire, Oregon State University, Corvallis, Oregon, *Adsorption, Proteins with Synthetic Materials*

Carol McLean, Protein Fractionation Centre, Scottish National Blood Transfusion Service, Edinburg, Scotland, *Contamination Detection in Animal Cell Culture*

Bernd Meibohm, College of Pharmacy, The University of Tennessee Health Science Center, Memphis, Tennessee, *Biopharmaceutics, Pharmacokinetics and Pharmacodynamics of Protein Therapeutics*

J.C. Merchuk, Ben-Gurion University of the Negev Beer-Sheva, Israel, *Bioreactors, Airlift Reactors*

J. Meriikhi, Philips Research Laboratories, Aachen, Germany, *Luminescent Materials, Biological Applications*

Otto-Wilhelm Merten, Généthon, Evry, France, *Cell Detachment*

Paul Messner, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

Hans-Peter Meyer, Lonza AG, Visp, Switzerland, *Industrial Biotransformation*

B. Micheel, University of Potsdam, Institute of Biochemistry and Biology, Potsdam, Germany, *Animal Cell Types, Hybridoma Cells*

George Miesegaes, Office of Biotech Products, Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, *Virus Retentive Filters*

Wolfgang Minas, Biotech Concepts Ltd, Zürich, Switzerland, *Erythromycins*

Shelley D. Minteer, Saint Louis University, St. Louis, Missouri, *Biofuel Cells, Enzyme Catalysis of Oils*

Manuel Miró, University of the Balearic Islands Carretera de Valldemossa, Illes Balears, Spain, *Flow Injection Analysis (FIA)*

David A. Mitchell, Universidade Federal do Parana, Curitiba, Brazil, *Solid State Fermentation, Microbial Growth Kinetics*

Inger Mollerup, Novo Nordisk A/S, Bagsvaerd, Denmark, *Insulin Purification*

Jamie Moore, Pharmaceutical Development Genentech, Inc., South San Francisco, California, *Freezing, Biopharmaceutical Products*

Eric Morello, Unité Biologie Moléculaire du Gène chez les Extrêmophiles, Institut Pasteur, Paris, France, *Secretion of Heterologous Proteins, Gram-Positive Bacteria, Lactococcus lactis*

Sherie L. Morrison, Immunology and Molecular Genetics, University of California, Los Angeles, California, *Antibody Engineering*

Manuel Mota, IBB, Centro de Eng. Biológica, University of Minho, Portugal, *Porous Media, Applications in Biotechnology*

Arun S. Mujumdar, National University of Singapore, Singapore, *Drying of Biological Materials*

Ashim Mullick, Unilever R&D, Hindustan Unilever Research Centre, Bangalore, India, *Tea Bioactives*

Alaka Mullick, Institut de recherche en biotechnologie, Conseil national de recherches du Canada, Montréal, Québec, Canada, *Transcription, Translation, and the Control of Gene Expression in Mammalian Cells*

David W. Murhammer, University of Iowa, Iowa City, Iowa, *Pluronic Polyols, Cell Protection*

Vanessa Murphy, Waterford Institute of Technology, Waterford, Ireland, *Biosorption, Metals*

J. Colin Murrell, University of Warwick, Coventry, United Kingdom, *Methanotrophs*

Mariam Naciri, University College Dublin, Belfield, Dublin, Ireland, *Cell Cycle Synchronization; Cell Cycle, Importance in Bioprocesses*

Yuri Nagai, Fermentation and Biotechnology Laboratories, Ajinomoto Co, Inc., Kawasaki, Japan, *Amino Acid Production,* L-*Lysine*

Ghinwa Melodie Naja, McGill University, Montreal, Canada, *Biosorption, Metals*

Toru Nakayama, Tohoku University, Sendai, Japan, *β-Galactosidase*

Viswaprakash Nanduri, Molecular Food Microbiology Laboratory, Purdue University, West Lafayatte, Indiana, *Biosensors, Foodborne Pathogen Detection*

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Someet Narang, Johns Hopkins University, Baltimore, Maryland; MedImmune, Inc. Gaithersburg, Maryland, *Insect Cells and Larvae, Gene Expression Systems*

Bhagyalakshmi Neelwarne, Central Food Technological Research Institute, Mysore-India, *Monocots, Tissue Culture*

Nuttawee Niamsiri, Faculty of Science, Mahidol University, Bangkok, Thailand, *Bionanofabrication of Nanostructures and Functional Materials*

Lars Keld Nielsen, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia, *Baculovirus Kinetics in Insect Cell Culture*

Jens Nielsen, Technical University of Denmark, Lyngby, Denmark, *Fermentation Monitoring*

Alvin W. Nienow, University of Birmingham, School of Chemical Engineering, Birmingham, United Kingdom, *Impeller Selection for Animal Cell Culture; Scale-Up, Stirred Tank Reactors*

Yasuo Ninomiya, Nitto Denko Company, Osaka, Japan, *Insecticides, Microbial Production; Optical Resolution, Biocatalysis*

Takuo Nishida, Tanabe Seiyaku Co., Ltd., Osaka, Japan, *Diltiazem Synthesis, Microbial Asymmetric Reduction*

P.T. Noble, Fluor Daniel GmbH, Wiesbaden, Germany, *Sterilization-in-Place*

Jeffery Odum, Principal, NC Biosource, Hokkaido University, North Carolina, *Closed Systems in Bioprocessing*

Jun Ogawa, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, *Oils, Microbial Production*

Kirsi-Marja Oksman-Caldentey, Technical Research Centre of Finland (VTT), Espoo, Finland, *Culture Establishment, Plant Cell*

Lisbeth Olsson, Chalmers University of Technology, Industrial Biotechnology, Göteborg, Sweden, *Beer Brewing, Applications of Metabolic Engineering*

Gustaf Olsson, Lund University, Lund, Sweden, *Water and Wastewater Treatment, Automation*

Tetso Omata, Nitto Denko Company, Osaka, Japan, *Insecticides, Microbial Production; Optical Resolution, Biocatalysis*

Shohei Ookino, Research Institute of Innovative Technology for the Earth, Kyoto, Japan, *L-Aspartic Acid, Production Processes*

Maryna Ornatska, Clarkson University, Potsdam, New York, *Biosensors, Toxicity Monitoring*

Gabriel Padilla, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, QLD, Australia; Institute of Biomedical Sciences, University of SÃo Paulo, SÃo Paulo, SP, Brazil, *Anticancer Compounds Development, Structural Modifications and Therapeutic Applications*

R. Palanisamy, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications*

Laura A. Palomares, Universidad Nacional Autónoma de México, Cuernavaca, México, *Bioreactor Scale-Up; Bioreactor Scale-Down*

Nicolai S. Panikov, Northeastern University, Boston, Massachusetts, *Kinetics, Microbial Growth*

Maria Papagianni, Aristotle University of Thessaloniki, Greece, *Rheology of Filamentous Microorganisms, Submerged Culture*

Victor Papavasileiou, Intelligen Europe, Leiden, The Netherlands, *Bioprocess Design, Computer-Aided*

Nádia Skorupa Parachin, Lund University, Lund, Sweden, *Bioreduction*

Nancy L. Parenteau, Parenteau BioConsultants LLC Fair Haven, Vermont, *Cell Differentiation, In vitro, Mammalian*

Jun T. Park, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, United States Food and Drug Administration, *Quality by Design (QbD), Biopharmaceutical Manufacture*

Jussi Parkkinen, Lappeenranta University of Technology, Lappeenranta, Finland, *Biosensors, Color-Sensitive*

Sinikka Parkkinen, Lappeenranta University of Technology, Lappeenranta, Finland, *Biosensors, Color-Sensitive*

N. Parlane, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand; Hopkirk Research Institute, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications*

Ramesh N. Patel, SLRP Associates, Biotechnology Consultation, Bridgewater, New Jersey, *Enzymatic Synthesis of Chiral Drug Intermediates*

K. Peter Pauls, University of Guelph, Guelph, Ontario, Canada, *Toxin Resistant Plants from Plant Cell Culture and Transformation*

Manuel L. Penichet, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California, *Monoclonal Antibodies, Human, Engineered*

Michel Penninckx, Faculté des Sciences–Ecole Interfacultaire des Bioingénieurs, Laboratoire de Physiologie et d'Ecologie Microbiennes (UPEM), Université libre de Bruxelles, c/o Institut de Santé Publique, Rue Engeland, Bruxelles–Belgium, *Crabtree Effect*

Steve Peppers, GE Healthcare Bio-Sciences AB, Uppsala, Sweden, *High-Throughput Technologies in Bioprocess Development*

Patrizia Perego, G.B. Bonino, University of Genoa, Genoa, Italy, *Microbiological Induced Corrosion and Inhibition*

Helene Perreault, University of Manitoba, Winnipeg, Manitoba, Canada, *Protein Glycosylation, Methods for Determination*

Adalberto Pessoa Jr., School of Pharmaceutical Sciences, University of São Paulo, Brazil, *Lipopolysaccharide, LPS removal, Depyrogenation*

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Demetri Petrides, Intelligen, Inc., Scotch Plains, New Jersey, *Bioprocess Design, Computer-Aided*

Urs Alexander Peuker, TU Bergakademie Freiberg, Institute for Mechanical Process Engineering and Mineral Processing, Freiberg, Germany, *Bioseparation, Magnetic Particle Adsorbents*

Delphine D. Le Pevelen, Chiralabs Ltd., Begbroke Centre for Innovation and Enterprise, Oxford University Begbroke Science Park, Yarnton (Oxfordshire), UK, *Spectral Data Comparability–Acceptance Criteria Definition by Statistical Analysis*

Theresa B. Phamduy, Rensselaer Polytechnic Institute, Troy, New York, *Bioprinting*

Lauren B. Pickens, University of California Los Angeles, Los Angeles, California, *Antibiotics, Polyketide*

Maya Piddocke, Center for Microbial Biotechnology, Technical University of Denmark, Lyngby, Denmark, *Beer Brewing, Applications of Metabolic Engineering*

John Pieracci, Biogen Idec, San Diego, California, *Membrane Chromatography*

Mirella S. Pilone, Università degli studi dell'Insubria, Varese, Italy, *Enzymes,* D-*Amino Acid Oxidases*

Ouyang Pingkai, College of Life Science and Pharmacy, Nanjing University of Technology, Nanjing, China, L-*Malic Acid Production by Fumarase*

Tom Piombino, Integrated Project Services, Inc. Lafayette Hill, Lafayette Hill, Pennsylvania, *Single-Use (SU) Systems*

Tina Pitarresi, Fast Trak Biopharma Services, GE Healthcare, Piscataway, New Jersey, *Cleaning and Sanitization in Downstream Processing*

David Pollard, Merck & Co. Inc, Rahway, New Jersey, *Vent Gas Analysis*

Loredano Pollegioni, Centro Interuniversitario di Ricerca in Biotecnologie Proteiche "The Protein Factory", Politecnico di Milano and Università degli studi dell'Insubria, Milan, Italy, *Enzymes,* D-*Amino Acid Oxidases*

Laura Popolo, Università degli Studi di Milano, Milano, Italy, *Protein Synthesis and Secretion, Animal Cells*

Isabelle Poquet, Unité des Bactéries Lactiques et pathogènes Opportunistes (UR888), Jouy-en-Josas, France, *Secretion of Heterologous Proteins, Gram-Positive Bacteria, Lactococcus lactis*

Robert D. Possee, Centre for Ecology and Hydrology, Oxford, United Kingdom, *Baculovirus Expression Systems*

J.B. Power, University of Nottingham, School of Biosciences, Sutton Bonington Campus, Loughborough, United Kingdom, *Plant Protoplasts*

John E. Preece, Southern Illinois University, Carbondale, Illinois, *Acclimatization of Plantlets from In vitro to the Ambient Environment*

Gianluca Di Profio, Institute on Membrane Technology (ITM-CNR), University of Calabria, Arcavacata di Rende (CS), Italy, *Protein Crystallization, Kinetics*

Dietmar Pum, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

Mirjana Radosevich, Human Protein Process Sciences, Lille, France, *Affinity Chromatography—Fractionated and DNA-Engineered Plasma Proteins*

Octavio T. Ramírez, Universidad Nacional Autónoma de México, Cuernavaca, México, *Bioreactor Scale-Up; Bioreactor Scale-Down*

Margaret M. Ramsay, Royal Botanic Gardens, Richmond, Surrey, United Kingdom, *Dicotyledons*

Govind Rao, Bio-Manufacturing Sciences Group, Pfizer, New York, *Biominiaturization of Bioreactors*

I.A. Rasiah, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications*

Erik K. Read, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, United States Food and Drug Administration, *Quality by Design (QbD), Biopharmaceutical Manufacture*

Kenneth F. Reardon, Colorado State University, Fort Collins, Colorado, *Fluorescence Techniques for Bioprocess Monitoring*

Manfred T. Reetz, Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr, Germany, *Enzymes, Directed Evolution*

Jessica Rehdorf, Institute of Biochemistry, Enzyme Catalysis, Greifswald, Germany, *Monooxygenases, Baeyer-Villiger Applications in Organic Synthesis*

Bernd H.A. Rehm, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, *Biopolyester Particles: Preparation and Applications; Pseudomonas Applications*

Hans Reichenbach, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany, *Myxobacteria*

Steve Reid, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia, *Baculovirus Kinetics in Insect Cell Culture*

James J. Reilly, Laureate Pharma, Inc., Princeton, New Jersey, *Antibody Purification: Monoclonal (Mab) and Polyclonal*

Robert van Reis, Genentech, Inc., South San Francisco, California, *Protein Ultrafiltration*

Jong IL Rhee, Chonnam National University, GwangJu, Republic of Korea, *Immuno-Flow Injection Analysis (FIA), Process Monitoring and Control*

Grainne L. Riley, School of Chemical Engineering, University of Birmingham, United Kingdom, *Rheological Behavior of Fermentation Fluids*

Steven Ripp, University of Tennessee, Center for Environmental Biotechnology, Knoxville, Tennessee, *Bioelectronics*

Heiko Rischer, Technical Research Centre of Finland (VTT), Espoo, Finland, *Culture Establishment, Plant Cell*

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Anneli Ritala, Technical Research Centre of Finland (VTT), Espoo, Finland, *Culture Establishment, Plant Cell*

Peter L. Roberts, R&D Department, Bio Products Laboratory, Elstree, Hertfordshire, United Kingdom, *Sterilization and Decontamination, Bioprocess Equipment and Biological Products*

Craig Robinson, GE Healthcare, Westborough, Massachusetts, *Cleaning and Sanitization in Downstream Processing*

David K. Robinson, Bioprocess Research & Development, Merck Research Laboratories, Rahway, NJ, *Monoclonal Antibody Production, Cell Lines*

Carl A. Rockburne, The Rockburne Group, Atlanta, Georgia, *Chromatography, Industrial Scale, Validation*

Angela Rodgers, National Institute for Biological Standards and Control, South Mimms, England, *Animal Cell Types, B Cell Lineage; Animal Cell Types, Monocyte, Macrophage and Dendrite Lineage*

Liliana Rodolfi, CNR, Istituto per lo Studio degli Ecosistemi, Firenze, Italy, *Photobioreactors*

José A. Rodríguez, University of California at Los Angeles, Los Angeles, California, *Monoclonal Antibodies, Human, Engineered*

Marta Rodríguez-Sáiz, R&D Biology, Antibióticos S. A., León, Spain, *Cephalosporin Production by Fungal Metabolic Engineering*

Gustav Rodrigo, GE Healthcare Bio-Sciences AB, Uppsala, Sweden, *High-Throughput Technologies in Bioprocess Development*

Palmer Rogers, University of Minnesota, Minneapolis, Minnesota, *Clostridia, Solvent Formation*

Rodica-Viorica Roman, Chemical Pharmaceutical Research Institute, Iaşi, Romania, *Transfer Phenomena in Multiphase Systems*

Cees Ronda, Philips Research Laboratories, Aachen, Germany; Utrecht University, Utrecht, The Netherlands; Zhejiang University, Hangzhou, China, *Luminescent Materials, Biological Applications*

Cécile Roux, UPMC Univ Paris 6, CNRS and Collège de France, Chimie de la Matière Condensée de Paris, Paris, France, *Biocers, Industrial Applications*

Antoni Sánchez, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain, *Dehydrogenase*

José-María Sánchez-Montero, Facultad de Farmacia, Universidad Complutense, Madrid, Spain, *Enzyme-Catalyzed Synthesis of Nonnatural or Modified Nucleosides*

Eiji Sakuradani, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, *Oils, Microbial Production*

W. Mark Saltzman, Cornell University, Ithaca, New York, *Extracellular Matrix and Cell Adhesion*

Sergio Sanchez, Universidad Nacional Autónoma de México, México D.F., Mexico, *Biosynthesis of Microbial Primary Metabolites*

Cesar C. Santana, School of Chemical Engineering, State University of Campinas, Campinas-SP, Brazil, *Adsorption in Simulated Moving Beds*

Ryuzo Sasaki, Kyoto University, Kyoto, Japan, *Erythropoietin*

Tadashi Sato, Tanabe Seiyaku Company, Ltd., Osaka, Japan, *L-Amino Acids Production by Aminoacylase*

Rachel Saunders, School of Materials, Materials Science Centre, University of Manchester, United Kingdom, *Bioprinting, Inkjet Deposition*

Maria Schäfer, TU Bergakademie Freiberg, Institute for Mechanical Process Engineering and Mineral Processing, Freiberg, Germany, *Bioseparation, Magnetic Particle Adsorbents*

Christina Schäffer, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

Catherine H. Schein, Sealy Center for Structural Biology and Molecular Biophysics, Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, Texas, *Protein Aggregation and Precipitation, Measurement and Control; Soluble Protein Expression in Bacteria*

Thomas Scheper, Institut für Technische Chemie, Gottfried Wilhelm Leibniz Universität Hannover, Hannover, Germany, *Fluorescence Techniques for Bioprocess Monitoring*

Bettina Schiel, Institut für Mikrobiologie und Biotechnologie, Universität Ulm, Ulm, Germany, *Clostridium*

J.E. Schlatmann, Delft University for Technology, The Netherlands, *Recirculation Bioreactor in Plant Cell Culture*

Claudia Schmidt-Dannert, University of Minnesota, St. Paul, Minnesota, *Carotenoids, Microbial Processes*

Ole Schou, Novo Nordisk A/S, Bagsvaerd, Denmark, *Insulin Purification*

Bernhard Schuster, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

Henry Schwartzberg, University of Massachusetts, Amherst, Massachusetts, *Food Process Engineering*

Alan Scragg, University of the West of England, Frenchay, Bristol, United Kingdom, *Plant Cell Cultures, Screening for Secondary Product Accumulation*

Richard Brent Seale, University of Otago, Dunedin, New Zealand, *Clean-in-Place (CIP)*

Jürgen Seibel, Institute for Chemical Engineering, Technical University, Braunschweig, Germany; Institut für Organische Chemie, Universität Würzburg, Am Hubland, Würzburg, Germany, *Enzymatic Oligosaccharide Synthesis; Glycochips*

Michael Seibert, National Renewable Energy Laboratory, Golden, Colorado, *Algal Hydrogen Production*

Klaus Selber, Heinrich Heine University Düsseldorf, Jülich, Germany, *Protein Purification, Aqueous Liquid Extraction*

Arindom Sen, Pharmaceutical Production Research Facility (PPRF), Schulich School of Engineering, University of Calgary, Alberta, Canada, *Neural Stem Cells, Bioprocess Engineering*

Shuji Senda, Nitto Denko Company, Osaka, Japan, *Insecticides, Microbial Production; Optical Resolution, Biocatalysis*

Gargi Seth, Genentech, Inc., 1 DNA Way, South San Francisco, California, *Mammalian Cell Bioreactors*

Francisco Gabriel Fernández Sevilla, University of Almería, Almería, Spain, *Microalgae, Mass Culture Methods*

Jose María Fernández Sevilla, University of Almería, Almería, Spain, *Microalgae, Mass Culture Methods*

Kevin L. Shade, Novartis Vaccines and Diagnostics, Speke, Liverpool, United Kingdom, *Aseptic Techniques in Cell Culture*

Rakhi B. Shah, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, United States Food and Drug Administration, *Quality by Design (QbD), Biopharmaceutical Manufacture*

Susan T. Sharfstein, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York, *Microcarrier Culture*

Robert Shawley, Late Stage Cell Culture, Genentech, Inc., San Francisco, California, *Animal Cell Culture Media*

Yin-Chu Shen, Institute of Bioengineering, Zhejiang University of Technology, Hanghzou, China, *Acrylamide, Microbial Production by Nitrile Hydratase*

John L. Sherwood, University of Georgia, Athens, Georgia, *Virus Removal from Plants*

Li Shi, Genzyme, Framingham, Massachussets, *Proteins, Thermal Unfolding*

Takeji Shibatani, Tanabe Seiyaku Co., Ltd., Osaka, Japan, *Diltiazem Synthesis; Diltiazem Synthesis, Microbial Asymmetric Reduction*

Sakayu Shimizu, Kyoto University, Kyoto, Japan, *Aldehyde Reductase; Lactonohydrolase; Oils, Microbial Production; Pantothenic Acid and Related Compounds*

Bryan Shingle, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

Suteaki Shioya, Sojo University, Graduate School of Engineering, Kumamoto, Japan, *Mixed Culture Processes*

Masaru Shiratori, Late Stage Cell Culture, Genentech, Inc., San Francisco, California, *Animal Cell Culture Media*

Joan Qi Si, Novo Nordisk Ferment Ltd., Dittingen, Switzerland, *Enzymes, Baking Processes*

Christian Sieblist, Institute of Biotechnology; Centre of Bioengineering, Martin-Luther-University Halle-Wittenberg, Weinbergweg, Halle (Saale), Germany, *Gas Holdup in Bioreactors*

Johannes Siemens, Technische Universitat Dresden, Dresden, Germany, *Genetic Transformation, Plants*

Yajaira Sierra-Sastre, Cornell University, Ithaca, New York, *Bionanofabrication of Nanostructures and Functional Materials*

Charles Siletti, Intelligen, Inc., Mt. Laurel, New Jersey, *Bioprocess Design, Computer-Aided*

Ivanildo J. Silva Jr., Federal University of Ceará, Fortaleza-CE, Brazil, *Adsorption in Simulated Moving Beds*

Carla Silva, Universidade do Minho, Braga, Portugal, *Biotransformation of Synthetic Fibers*

Martin S. Sinacore, Genetics Institute, Andover, Massachusetts, *Cell Stability, Animal*

Rabinder P. Singh, University College Dublin, School of Chemical and Bioprocess Engineering, Belfield, Ireland, *Apoptosis*

Satish K. Singh, Pharmaceutical Research and Development, Global Biologics, Pfizer Global Research and Development, Chesterfield, Missouri, *Temperature-Dependent Molecular Adaptations, Microbial Proteins*

Jose-Vicente Sinisterra, Servicio de Biotransformaciones Industriales, Parque Científico de Madrid, Madrid, Spain; Facultad de Farmacia, Universidad Complutense, Madrid, Spain, *Enzyme-Catalyzed Synthesis of Nonnatural or Modified Nucleosides*

A.J. Sinskey, Massachusetts Institute of Technology, Cambridge, Massachusetts, *Corynebacteria, Brevibacteria*

Uwe B. Sleytr, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Austria, *S-layers, Microbial, Biotechnological Applications*

Greg Van Slyke, Invitrogen, part of Life Technologies, Grand Island, New York, *High-Throughput Technologies in Bioprocess Development*

David Smith, CAB International Europe UK, Egham, UK, *Culture Collections and Biological Resource Centers (BRCs)*

Thomas J. Smith, Biomedical Research Centre, Sheffield Hallam University, Sheffield, United Kingdom, *Methanotrophs; Monooxygenases, Bacterial, Oxidation of Alkenes*

Mark E.B. Smith, University College London, London, United Kingdom, *Transketolases*

Sharon Smolinski, National Renewable Energy Laboratory, Golden, Colorado, *Algal Hydrogen Production*

Leo Snel, Novo Nordisk A/S, Bagsvaerd, Denmark, *Insulin Purification*

Eduardo V. Soares, Bioengineering Laboratory, Superior Institute of Engineering from Porto Polytechnic Institute, Porto, Portugal; IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Braga, Portugal, *Cell Separation: Yeast Flocculation*

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Gail Sofer, GE Healthcare, Piscataway, New Jersey, *Cleaning and Sanitization in Downstream Processing*

Benjamin Sommer, University of Bielefeld, Faculty of Technology, Fermentation Engineering, Bielefeld, Germany, *Cell Viability, Measurement*

Yuan-Quan Song, Tsinghua University, Beijing, China, *1,2 and 1,3 Propanediol, Microbial Production Methods*

David D. Songstad, Monsanto, Saint Louis, Missouri, *Herbicide Tolerant Plants, Production*

Alexander Sorkin, University of Colorado Denver, Aurora, Colorado, *Growth Factors: Protein Processing, Endocytosis, and Intracellular Sorting*

Tanaka Soukichi, Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan, *Membrane-Surface Liquid Culture, Fungi*

Richard Sparling, University of Manitoba, Winnipeg, Manitoba, Canada, *Energy Metabolism of Cells Used for Industrial Production*

Raymond E. Spier, University of Surrey, Guildford, Surrey, United Kingdom, *Ethical Issues in Animal and Plant Cell Technology; History of Animal Cell Technology*

Koti Sreekrishna, Procter & Gamble Co., Cincinnati, Ohio, *Pichia, Optimization of Protein Expression*

Friedrich Srienc, BioTechnology Institute, University of Minnesota, Minnesota, *Enzyme-Catalyzed Reactions in Ionic Liquids*

Haralambos Stamatis, University of Ioannina, Ioannina, Greece, *Reverse Micelles, Enzymes*

Winfried Storhas, Mannheim University of Applied Sciences, Mannheim, Germany, *Bioreactors, Fluidized-Bed*

Bob Stover, Tri-Clover, Valencia, California, *Pumps, Industrial*

Peter J. Strong, SCION Rotorua, New Zealand, *Laccase*

Deidre M. Stuart, Queensland University of Technology, Brisbane, Australia, *Solid State Fermentation, Microbial Growth Kinetics*

Wei Wen Su, University of Hawaii, Honolulu, Hawaii, *Bioreactors, Perfusion*

Zhi-Guo Su, National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China, *Enzyme Immobilization, Biocatalyst Featured with Nanoscale Structure*

Shyamsundar Subramanian, Merck & Co., Inc., West Point, Pennsylvania, *Animal Cells, Hybridomas, Human Antibody Production*

M. Sugimoto, Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan, *Amino Acids, Production Processes*

Masaru Suto, Nitto Chemical Industry Co., Ltd., Tokyo, Japan, *Nitrile Hydratase*

T. Swaminathan, Indian Institute of Technology–Madras, Chennai, India, *Dextran, Microbial Production*

Christoph Syldatk, Institut für Bio-und Lebensmitteltechnik, Bereich II: Technische Biologie, Universität Karlsruhe (TH), Engler-Bunte-Ring 1, Karlsruhe, Germany, *Biosurfactants, Rhaminolipid, Microbial Production*

Satomi Takahashi, Kaneka Corporation, Hyogo, Japan, *Aminohydrolases for Production of* D-*Amino Acids;* D-*Phenylglycines*

Atsuo Tanaka, Kyoto University, Kyoto, Japan, *Organosilicon Compounds, Enzymatic Modification*

Yi Tang, University of California Los Angeles, Los Angeles, California, *Antibiotics, Polyketide*

Robert D. Tanner, Vanderbilt University, Nashville, Tennessee, *Solid State Fermentation, Microbial Growth Kinetics*

Jörg Thömmes, Biogen Idec, San Diego, California, *Membrane Chromatography*

Jules Thibault, University of Ottawa, Ottawa, Canada, *Pullulan, Microbial Production Methods*

Kristina W. Thiel, Vanderbilt University School of Medicine, Nashville, Tennessee, *Cell-Surface Receptors: Structure, Activation, and Signaling*

Jacques Thierie, Faculté des Sciences–Ecole Interfacultaire des Bioingénieurs, Laboratoire de Physiologie et d'Ecologie Microbiennes (UPEM), Université libre de Bruxelles, c/o Institut de Santé Publique, Rue Engeland, Bruxelles–Belgium, *Crabtree Effect*

Colin R. Thomas, School of Chemical Engineering, University of Birmingham, United Kingdom, *Rheological Behavior of Fermentation Fluids*

Owen Thomas, University of Birmingham, Biochemical Engineering, Birmingham, United Kingdom, *Bioseparation, Magnetic Particle Adsorbents*

Claudio Thomasin, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

Paul B. Thompson, Michigan State University—Philosophy, Michigan, *Agricultural Biotechnology, Food Safety, Risk, Consent, and Ethics*

J.S. Tolan, Iogen Corporation, Ottawa, Canada, *Enzymes, Pulp and Paper Processing*

Marcelo E. Tolmasky, California State University, Fullerton, Fullerton, California, *Plasmid DNA Replication*

Tetsuya Tosa, Tanabe Seiyaku Company, Ltd., Osaka, Japan, L-*Amino Acids Production by Aminoacylase*

Annabella Tramice, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Napoli, Italy, *Enzymatic Transglycosylation*

Johannes Tramper, Food and Bioprocess Engineering Group, Wageningen University, The Netherlands, *Flux Analysis of Mammalian Cell Culture*

George E. Tranter, Chiralabs Ltd., Begbroke Centre for Innovation and Enterprise, Oxford University Begbroke Science Park, Yarnton (Oxfordshire), UK, *Spectral Data Comparability–Acceptance Criteria Definition by Statistical Analysis*

Mario R. Tredici, Università degli Studi di Firenze, Firenze, Italy, *Photobioreactors*

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Jorge Trelles, Universidad Nacional de Quilmes, Buenos Aires, Argentina, *Enzyme-Catalyzed Synthesis of Nonnatural or Modified Nucleosides*

Antonio Trincone, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Napoli, Italy, *Enzymatic Transglycosylation*

Radu Z. Tudose, Technical University Gh.Asachi Iaşi, Iaşi, Romania, *Static Mixing in Fermentation Processes*

Benedetta Turchetti, University of Perugia, Perugia, Italy, *Basidiomycetous Yeasts for Production of Carotenoids*

Anthony P.F. Turner, Cranfield Health, Cranfield University, Bedfordshire, United Kingdom, *Biosensors, Aptamers (Aptasensors), Bioprocess Monitoring*

Richard M. Twyman, John Inness Centre, Norwich, United Kingdom, *Genetic Engineering, Animal Cell*

Makoto Ueda, Mitsubishi Chemical Co., Yokohama, Japan, D-*Malate*

Michiel E. Ultee, Laureate Pharma, Inc., Princeton, New Jersey, *Antibody Purification: Monoclonal (Mab) and Polyclonal*

Cord C. Uphoff, DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, *Mycoplasma Contamination Of Cell Cultures*

Pavan K. Vaddady, The University of Tennessee Health Science Center, Memphis, Tennessee, *Biopharmaceutics, Pharmacokinetics and Pharmacodynamics of Protein Therapeutics*

Patricia S. Vary, Northern Illinois University, DeKalb, Illinois, *Bacillus Megaterium and Other Bacilli: Industrial Applications*

María C. Veiga, Chemical Engineering Laboratory, Faculty of Sciences, University of La Coruña, La Coruña, Spain, *Biofilters for Air Purification*

Richard E. Veilleux, Virginia Polytechnic Institute & State University, Blacksburg, Virginia, *Haploid Plant Production, Pollen, Anther, and Ovule Culture*

Rami Reddy Vennapusa, Downstream Bioprocessing Laboratory, School of Engineering and Science, Jacobs University, Bremen, Germany, *Expanded Bed Chromatography, Surface Energetics of Biomass Deposition*

Manojlović Verica, University of Belgrade, Belgrade, Republic of Serbia, *Immobilized Cells*

Madan Lal Verma, H. P. University, Summer Hill, Shimla, *Lipases*

Luciana De Vero, University of Modena and Reggio Emilia, Reggio Emilia, Italy, *Acetic Acid Bacteria, Biotechnological Applications*

Natarajan Vijayasankaran, Late Stage Cell Culture, Genentech, Inc., San Francisco, California, *Animal Cell Culture Media*

Nedović Viktor, University of Belgrade, Belgrade, Republic of Serbia, *Immobilized Cells*

Bohumil Volesky, McGill University, Montreal, Canada, *Biosorption, Metals*

Lawrence P. Wackett, BioTechnology Institute, St. Paul, Minnesota, *Database Tools for Microbial Biocatalysis*

Masaru Wada, Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, *Central Metabolism Enhancement by Defective F_1F_o-ATP Synthase*

Kenneth Barry Walker, National Institute for Biological Standards and Control, South Mimms, England, *Animal Cell Types, B Cell Lineage; Animal Cell Types, Monocyte, Macrophage and Dendrite Lineage*

Gary Walsh, Industrial Biochemistry and Materials Surface Sciences Institute, University of Limerick, Limerick City, Ireland, *Regulatory Requirements for Human Biopharmaceuticals, European Community*

Vanessa Walter, Institut für Bio-und Lebensmitteltechnik, Bereich II: Technische Biologie, Universität Karlsruhe (TH), Engler-Bunte-Ring 1, Karlsruhe, Germany, *Biosurfactants, Rhaminolipid, Microbial Production*

Wei Wei Wang, The University of Western Ontario, Mechanical and Materials Engineering, London, Ontario, Canada, *Biophotoreceptor Arrays*

Ping Wang, Biotechnology Institute, University of Minnesota, St Paul, Minnesota, *Enzyme Immobilization, Biocatalyst Featured with Nanoscale Structure*

Owen P. Ward, University of Waterloo, Waterloo, Canada, *Bioremediation*

Dave A. Wareheim, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

James C. Warren, Merck & Co., Inc., West Point, Pennsylvania, *Animal Cells, Hybridomas, Human Antibody Production*

Kazuya Watanabe, Hashimoto Light Energy Conversion Project, ERATO/JST, Tokyo, Japan; The University of Tokyo, Research Center for Advanced Science and Technology, Tokyo, Japan, *Biofuel Cells, Performance Characterization*

Pamela J. Weathers, Biology and Biotechnology Department, Worcester Polytechnic Institute, Worcester, Massachusetts, *Bioreactors, Mist*

Tilmann Weber, Eberhard-Karls-Universität Tübingen, Mikrobiologie/Biotechnologie, Tübingen, Germany, *Antibiotics: Biosynthesis, Generation of Novel Compounds*

Ning Wei, University of Bielefeld, Faculty of Technology, Fermentation Engineering, Bielefeld, Germany, *Cell Viability, Measurement*

Sandy Weinberg, Clayton State University, Atlanta, Georgia, *Chromatography, Industrial Scale, Validation*

Rick Welsh, Clarkson University, New York, *Agricultural Biotechnology and Socioeconomic Effects*

David White, University Bloomington, Indiana, *Electron Transport*

Bruce Whitelaw, Roslin Institute, Roslin, Midlothian, United Kingdom, *Genetic Engineering, Animal Cell*

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Erik M. Whiteley, Geron Corporation, Menlo Park, California, *Insect Cells and Larvae, Gene Expression Systems*

Jack M. Widholm, University of Illinois at Urbana-Champaign, Urbana, *Plant Cell Cultures, Photosynthetic*

Ronnie Willaert, Flanders Interuniversity Institute for Biotechnology, Vrije Universiteit Brussel, Brussels, Belgium, *Cell Immobilization, Engineering Aspects*

L.B. Willis, Massachusetts Institute of Technology, Cambridge, Massachusetts, *Corynebacteria, Brevibacteria*

Jack Wilson, ABEC, Inc., Allentown, Pennsylvania, *Fermenter Design*

Roland Wohlgemuth, Research Specialties, Sigma-Aldrich GmbH, Buchs, Switzerland, *Industrial Biotransformation; Transketolases*

Christian Wood, Centocor R&D Spring House, Pennsylvania, *Single-Use (SU) Systems*

David W. Wood, The Ohio State University, Columbus, Ohio, *Large-Scale Protein Purification, Self-Cleaving Aggregation Tags*

John M. Woodley, Center for BioProcess Engineering, Technical University of Denmark, Lyngby, Denmark, *Transketolases*

Florian M. Wurm, Institute of Bioengineering, Laboratory of Cellular Biotechnology, École Polytechnique Fédéral de Lausanne (EPFL), Lausanne, Switzerland, *Chinese Hamster Ovary Cells, Recombinant Protein Production; Transient Gene Expression in Mammalian Cells*

Barbara E. Wyslouzil, Chemical Engineering Department, Ohio State University, Columbus, Ohio, *Bioreactors, Mist*

Yun-Zhen Xu, Tsinghua University, Beijing, China, *1,2 and 1,3 Propanediol, Microbial Production Methods*

Jian-he Xu, Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China, *Biocatalysis, Cofactor Regeneration*

Hideaki Yamada, Mitsubishi Chemical Co., Yokohama, Japan, D-*Malate*

Keiko Yamada-Onodera, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Takayama, Ikoma, Japan, *Secondary Alcohols, Optically Active, Micorbial Production*

Yanto Yanto, School of Chemical and Biomolecular Engineering; Parker H. Petit Institute of Bioengineering and Biosciences, *Enzymes, Enolate Reductases "Old Yellow Enzyme"*

Hisashi Yasueda, Institute of Life Sciences, Ajinomoto Co., Inc., Kawasaki, Japan, *Amino Acid Production,* L-*Lysine*

Alexander Yelshin, Polotsk State University, Novopolotsk, Belarus, *Porous Media, Applications in Biotechnology*

Inna Yelshina, Polotsk State University, Novopolotsk, Belarus, *Porous Media, Applications in Biotechnology*

Atsushi Yokota, Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, *Central Metabolism Enhancement by Defective F_1F_o-ATP Synthase*

Kenzo Yokozeki, Industrial Microbiology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan, *Oils, Microbial Production*

Hu Yonghong, College of Life Science and Pharmacy, Nanjing University of Technology, Nanjing, China, L-*Malic Acid Production by Fumarase*

Esther M. Yoo, University of California, Los Angeles, California, *Antibody Engineering*

Young Je Yoo, School of Chemical and Biological Engineering, Seoul National University, Seoul, Korea, *Thermostable Proteins*

Jonathan Yourkin, GE Instruments, Boulder, Colorado, *Cleaning and Sanitization in Downstream Processing*

Jiujiang Yu, USDA/ARS, Southern Regional Research Center, New Orleans, Louisiana, *Aflatoxins*

Hideaki Yukawa, Research Institute of Innovative Technology for the Earth, Kyoto, Japan, *Amino Acids, Branched Chain,* L-*Isoleucine; L-Aspartic Acid, Production Processes*

Jie Zhang, Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China, *Biocatalysis, Cofactor Regeneration*

David (Xiaojian) Zhao, Invitrogen, part of Life Technologies, Grand Island, New York, *High-Throughput Technologies in Bioprocess Development*

Ren-Chao Zheng, Institute of Bioengineering, Zhejiang University of Technology, Hanghzou, China, *Acrylamide, Microbial Production by Nitrile Hydratase*

Yu-Guo Zheng, Institute of Bioengineering, Zhejiang University of Technology, Hanghzou, China, *Acrylamide, Microbial Production by Nitrile Hydratase*

Jian-Jiang Zhong, Key Laboratory of Microbial Metabolism, Ministry of Education, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai, China; State Key Laboratory of Bioreactor Engineering, School of Biotechnology, East China University of Science and Technology, Shanghai, China, *Bioreactors, Cell Culture, Commercial Production; Plant Cell Culture, Secondary Product Accumulation*

Weichang Zhou, Genzyme Corporation, Framingham, Massachusetts, *Mammalian Cell Bioreactors*

Xin Zhou, State Key Laboratory of Bioreactor Engineering, School of Biotechnology, East China University of Science and Technology, Shanghai, China, *Plant Cell Culture, Secondary Product Accumulation*

Tan-Che Zhou, Molecular Biochemical Engineering Group, Key Laboratory of Microbial Metabolism, Ministry of Education, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University,

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Shanghai, China, *Bioreactors, Cell Culture, Commercial Production*

Wen-Wen Zhou, Molecular Biochemical Engineering Group, Key Laboratory of Microbial Metabolism, Ministry of Education, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai, China, *Bioreactors, Cell Culture, Commercial Production*

Mario Zilli, University of Genoa, Genoa, Italy, *Biofilters for Air Purification*

Graziella Chini Zittelli, CNR, Istituto per lo Studio degli Ecosistemi, Firenze, Italy, *Photobioreactors*

Marko Zlokarnik, Chemical Process Engineering, University of Köln, Cologne, Germany, *Scale-Up of Chemical and Biotechnological Processes*

Min-Hua Zong, South China University of Technology, Guangzhou, China, *Organosilicon Compounds, Enzymatic Modification*

Andrew L. Zydney, The Pennsylvania State University, University Park, Pennsylvania, *Protein Ultrafiltration*

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PICHIA, OPTIMIZATION OF PROTEIN EXPRESSION

KOTI SREEKRISHNA
Procter & Gamble Co.,
Cincinnati, Ohio

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

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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, 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), *ADE1* (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

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"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.

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,

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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 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-^{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,

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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 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

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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 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

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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 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.

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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 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

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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 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

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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 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.

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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 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.

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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.

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

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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)

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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PILOT PLANTS, DESIGN AND OPERATION

BETH H. JUNKER
Bioprocess R&D, Merck Research Laboratories, Rahway, New Jersey

INTRODUCTION

Several diverse roles, objectives, and purposes are associated with a bioprocessing, biochemical, or biopharmaceutical pilot plant. These include, but are not limited to, the manufacture of early- and late-phase clinical supplies, process development (including process characterization, validation, and comparability (1)) for new products in the company pipeline, process improvement including technical support for existing products in the market, and production of biologically produced materials (which are not intended for the clinic) to support basic research efforts such as screening, assays, and disease mechanism elucidation. Despite desires to design pilot facilities for diverse differences in scale, products, processes, clinical phases, and capacity, limited capital and operating budgets force value engineering decisions about "must have" and "nice to have" capabilities (2). Unlike chemical pilot plants, pharmaceutical pilot plants are used not only to scale-up laboratory processes, but also to produce developmental quantities of chemicals (and biological substances) for safety, toxicological, and clinical studies (3). Thus, balancing of the demands for scale-up and development research with those for clinical production is a key dilemma for pilot plant operation (4). This tension has resulted in an increased recent attention to the development of scale-down models to mimic larger scale performance and thus reduce demands for pilot scale process development runs (5). Another challenge is to determine what proportion of the material requirements for each phase of a product's development has to be outsourced to contract manufacturing organizations (CMOs). Some CMOs are even targeting clinical-scale production clients, essentially building pilot plants for hire (6). The timing and reliability of clinical material production is critical as expensive and time-consuming clinical programs can be delayed, thus postponing product application filings, approvals, and launches (7). Although traditionally pilot plants have not manufactured material for sale, accelerated launch dates, deferred manufacturing facility capital commitments, and other areas of project uncertainty have

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Editors in Chief

Richard H. Baltz
CognoGen Biotechnology Consulting, Indianapolis, IN 46220

Julian E. Davies
University of British Columbia, Vancouver, British Columbia, V6R 1Z3 Canada

Arnold L. Demain
Charles A. Dana Research Institute (R.I.S.E.), Drew University, Madison, NJ 07940

Editors

Alan T. Bull
School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom

Beth Junker
Bioprocess Research and Development, Merck Research Laboratories, Rahway, NJ 07065

Leonard Katz
SynBERC - Synthetic Biology Engineering Research Center, University of California-Berkeley, Emeryville, CA 94608

Lee R. Lynd
Thayer School of Engineering, Dartmouth College, Hanover, NH 03755

Prakash Masurekar
Department of Plant Biology & Plant Pathology, School of Environmental and Biological Sciences, Rutgers University, New Brunswick, NJ 08901-8520

Christopher D. Reeves
Amyris Biotechnologies, Emeryville, CA 94608

Huimin Zhao
Departments of Chemical and Biomolecular Engineering, Chemistry, and Bioengineering, Institute for Genomic Biology, Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Contributors

CHARLES A. ABBAS
James R. Randall Research Center,
Archer Daniels Midland Co., Decatur, IL 62521

CARL B. ABULENCIA
Verenium Corporation, San Diego, CA 92121

SPIROS N. AGATHOS
Unit of Bioengineering (GEBI), Université Catholique de Louvain, Place Croix du Sud 2/19, B-1348, Louvain-la-Neuve, Belgium

DYLAN C. ALEXANDER
Cubist Pharmaceuticals, Inc., Lexington, MA 02421

ZHIQIANG AN
Epitomics, Inc., 863 Mitten Road, Burlingame, CA 94010

ADEYMA Y. ARROYO
Process Research & Development, Process Development Engineering Department, Genentech Inc., South San Francisco, CA 94080

YASUHISA ASANO
Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

RICHARD H. BALTZ
CognoGen Biotechnology Consulting, Indianapolis, IN 46220

WU LI BAO
James R. Randall Research Center,
Archer Daniels Midland Co., Decatur, IL 62521

ANDREAS BECHTHOLD
Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften, Pharmazeutische Biologie und Biotechnologie, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

KYLE E. BEERY
James R. Randall Research Center,
Archer Daniels Midland Co., Decatur, IL 62521

RAMUNAS BIGELIS
Biosynthetic Chemistry and Infectious Diseases, Pfizer, Pearl River, NY 10965

GERALD BILLS
Fundación MEDINA, Avenida Conocimiento s/n, Parque Tecnológico Ciencias de la Salud, Armilla, Granada 18100, Spain

WESLEY P. BLACK
Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061

MARITE BRADSHAW
Botulinum Toxins Laboratory, Dept. of Bacteriology, University of Wisconsin, 1550 Linden Dr., 6340 MSB, Madison, WI 53706

KATIE M. BRANSCUM
Natural Products Discovery Group, Department of Chemistry and Biochemistry, 620 Parrington Oval, Room 208, University of Oklahoma, Norman, OK 73019

MATTHEW CHASE
AMRI, 21 Corporate Circle, Albany, NY 12203

C. PERRY CHOU
Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

ROBERT H. CICHEWICZ
Natural Products Discovery Group, Department of Chemistry and Biochemistry, 620 Parrington Oval, Room 208, University of Oklahoma, Norman, OK 73019

PATRICK C. CIRINO
Department of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802

DOUGLAS S. CLARK
Department of Chemical Engineering, University of California, Berkeley, 201 Gilman Hall, Berkeley, CA 94720

LOUIS A. CLARK
Codexis Inc., 200 Penobscot Drive, Redwood City, CA 94063

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PAMELA CORRINGTON
James R. Randall Research Center,
Archer Daniels Midland Co., Decatur, IL 62521

DON A. COWAN
Institute for Microbial Biotechnology and Metagenomics, Department of Biotechnology, University of the Western Cape, Bellville 7535, Cape Town, South Africa

JAMES M. CREGG
Keck Graduate Institute of Applied Life Sciences, Claremont, CA 91711

CONSUELO CRUZ
James R. Randall Research Center,
Archer Daniels Midland Co., Decatur, IL 62521

SAMUN K. DAHOD
Valent BioSciences Corp., Libertyville, IL 60048

STEFANO DONADIO
KtedoGen, Via Fantoli 16/15, 20138 Milano, Italy, and NAICONS, Via Fantoli 16/15, 20138 Milano, Italy

WOUTER A. DUETZ
Enzyscreen, Biopartner Center Leiden,
Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

ALEXANDRU DUMITRACHE
Dept. of Chemistry and Biology, Ryerson University, 350 Victoria St., Toronto, Ontario, Canada, M5B 2K3

SLAVA S. EPSTEIN
Department of Biology, Northeastern University, Boston, MA 02115

DAWN T. ERIKSEN
Department of Chemical and Biomolecular Engineering and Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 600 South Mathews Ave., Urbana, IL 61801

GIOVANNA E. FELIS
Department of Science, Technology and Market of Vine and Wine, University of Verona, 37134 Verona, Italy

MANUEL FERRER
Institute of Catalysis, CSIC, Marie Curie 2, Campus Cantoblanco, 28049 Madrid, Spain

JENS C. FRISVAD
Center for Microbial Biotechnology, Department of Systems Biology, Building 221, Søltofts Plads, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

EKATERINA GAVRISH
Department of Biology, Northeastern University, Boston, MA 02115

PETER N. GOLYSHIN
School of Biological Sciences, Bangor University, Deiniol Road, LL57 2UW Gwynedd, United Kingdom

OLGA V. GOLYSHINA
School of Biological Sciences, Bangor University, Deiniol Road, LL57 2UW Gwynedd, United Kingdom

MICHAEL GOODFELLOW
School of Biology, University of Newcastle, Newcastle upon Tyne, NE1 7RU, United Kingdom

KEVIN A. GRAY
Verenium Corporation, San Diego, CA 92121

RANDOLPH GREASHAM
Independent Consultant, 460 Bayberry Lane, Mountainside, NJ 07092

MELINDA J. GRIFFITHS
Centre for Bioprocess Engineering Research (CeBER), Department of Chemical Engineering, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

MARIA-EUGENIA GUAZZARONI
Institute of Catalysis, CSIC, Marie Curie 2, Campus Cantoblanco, 28049 Madrid, Spain

SUSAN T. L. HARRISON
Centre for Bioprocess Engineering Research (CeBER), Department of Chemical Engineering, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

JON C. HENRIKSON
Natural Products Discovery Group, Department of Chemistry and Biochemistry, 620 Parrington Oval, Room 208, University of Oklahoma, Norman, OK 73019

WILLIAM G. HERRICK
Dept. of Chemical Engineering, University of Massachusetts-Amherst, 686 N. Pleasant St., Amherst, MA 01003

LUTZ HILTERHAUS
Institute of Technical Biocatalysis, University of Technology Hamburg-Harburg, D-21071 Hamburg, Germany

DAVID A. HOGSETT
Mascoma Corporation, Lebanon, NH 03655

FLORIAN HOLLFELDER
Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, United Kingdom

DAVID HOPKINS
BioProcess Engineering, Genzyme Corporation, Framingham, MA 01701

WEI-SHOU HU
Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN 55455

ELTON P. HUDSON
Department of Chemical Engineering, University of California, Berkeley, 201 Gilman Hall, Berkeley, CA 94720

MASATO IKEDA
Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan

THOMAS W. JEFFRIES
USDA, Forest Service, Forest Products Laboratory and Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53726

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SUSAN E. JENSEN
Dept. of Biological Sciences, University of Alberta, Edmonton, AB Canada T6G 2E9

ERIC A. JOHNSON
Botulinum Toxins Laboratory, Dept. of Bacteriology, University of Wisconsin, 1550 Linden Dr., 6303 MSB, Madison, WI 53706

BRYAN JULIEN
Allylix, Inc., 1500 Bull Lea Rd., Lexington, KY 40511

PAVAN K. R. KAMBAM
Dept. of Chemical Engineering, University of Massachusetts-Amherst, 686 N. Pleasant St., Amherst, MA 01003

ANNE KANTARDJIEFF
Alexion Pharmaceuticals, Cheshire, CT 06410

JAMES T. KEALEY
Amyris Biotechnologies, 5885 Hollis St., Suite 100, Emeryville, CA 94608

JAY D. KEASLING
Joint BioEnergy Institute (JBEI), Physical Biosciences Division, Berkeley National Laboratory; Dept. of Chemical Engineering, Dept. of BioEngineering, Berkeley Center for Synthetic Biology (SynBERC), University of California, Berkeley, CA 94720

MARTIN KELLER
Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6026

MAX KENNEDY
Biolighthouse Ltd., Wellington, New Zealand

WAN-SEOP KIM
Bristol-Myers Squibb Company, 6000 Thompson Road, East Syracuse, NY 13057

BRONWYN M. KIRBY
Institute for Microbial Biotechnology and Metagenomics, Department of Biotechnology, University of the Western Cape, Bellville 7535, Cape Town, South Africa

JOEL A. KREPS
Verenium Corporation, 4955 Directors Place, San Diego, CA 92121

NICHOLAS LANGLEY
Centre for Bioprocess Engineering Research (CeBER), Department of Chemical Engineering, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

KIM LEWIS
Department of Biology, Northeastern University, Boston, MA 02115

ANDREAS LIESE
Institute of Technical Biocatalysis, University of Technology Hamburg-Harburg, D-21071 Hamburg, Germany

MICHAEL J. LISZKA
Department of Chemical Engineering, University of California, Berkeley, 201 Gilman Hall, Berkeley, CA 94720

LUCAS LOVELESS
James R. Randall Research Center, Archer Daniels Midland Co., Decatur, IL 62521

LEE R. LYND
Thayer School of Engineering and Dept. of Biological Sciences, Dartmouth College, Hanover, NH 03755

R. SCOTT MCIVOR
Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455

TRACY L. MEIRING
Institute for Microbial Biotechnology and Metagenomics, Department of Biotechnology, University of the Western Cape, Bellville 7535, Cape Town, South Africa

VERA MEYER
Institute of Biology, Department Molecular Microbiology and Biotechnology, Leiden University, 2333 BE Leiden, The Netherlands

JONATHAN R. MIELENZ
BioEnergy Science Center, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

UFFE H. MORTENSEN
Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

NIKHIL U. NAIR
Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, 600 South Mathews Ave., Urbana, IL 61801

KIEN T. NGUYEN
Cubist Pharmaceuticals, Inc., Lexington, MA 02421

DOMINICA NICHOLS
Department of Biology, Northeastern University, Boston, MA 02115

JENS NIELSEN
Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-41296 Gothenburg, Sweden

AHARON OREN
Department of Plant and Environmental Sciences, Institute of Life Sciences, and the Moshe Shilo Minerva Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

NANCY L. PAIVA
Dept. of Chemistry, Computer and Physical Sciences, Southeastern Oklahoma State University, Durant, OK 74701

NICOLAI S. PANIKOV
Thayer School of Engineering, Dartmouth College, 8000 Cummings Hall, Hanover, NH 03755

CHRISTOPHER J. PETZOLD
Joint BioEnergy Institute (JBEI), Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

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JACK PRIOR
BioProcess Engineering, Genzyme Corporation, Framingham, MA 01701

KATHARINA PROBST
Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften, Pharmazeutische Biologie und Biotechnologie, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

PETER J. PUNT
TNO Quality of Life, 3700 AJ Zeist, The Netherlands

MICHAEL E. PYNE
Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

ARTHUR F. J. RAM
Institute of Biology, Department Molecular Microbiology and Biotechnology, Leiden University, 2333 BE Leiden, The Netherlands

MANFRED T. REETZ
Department of Synthetic Organic Chemistry, Max-Planck-Institut für Kohlenforschung, 45476 Mülheim an der Ruhr, Germany

URSULA RINAS
Helmholtz Center for Infection Research, Inhoffenstr. 7, D-38124 Braunschweig, Germany

EDUARDO RODRIGUEZ
Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET) and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK, Rosario, Argentina

DANIEL J. SAYUT
Dept. of Chemical Engineering, University of Massachusetts-Amherst, 686 N. Pleasant St., Amherst, MA 01003

GARGI SETH
Genentech, Inc., South San Francisco, CA 94080

JOSEPH SHILOACH
Biotechnology Core Laboratory, National Institute of Diabetic, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

VERENA SIEWERS
Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-41296 Gothenburg, Sweden

MARGHERITA SOSIO
KtedoGen, Via Fantoli 16/15, 20138 Milano, Italy, and NAICONS, Via Fantoli 16/15, 20138 Milano, Italy

MARTIN SPARKS
James R. Randall Research Center, Archer Daniels Midland Co., Decatur, IL 62521

MELISSA ST. AMAND
Dept. of Chemical Engineering, University of Delaware, Newark, DE 19711

ULRICH STRYCH
Dept. of Biology and Biochemistry, University of Houston, 4800 Calhoun Rd., Houston, TX 77204-5001

KARAN S. SUKHIJA
Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

JIBIN SUN
Chinese Academy of Sciences, Tianjin Institute of Industrial Biotechnology, 300308 Tianjin, China, and Hamburg University of Technology, Institute of Bioprocess and Biosystems Engineering, Denickestr. 15, 21073 Hamburg, Germany

LIANHONG SUN
Dept. of Chemical Engineering, 221 Goessmann Lab, University of Massachusetts-Amherst, 686 N. Pleasant St., Amherst, MA 01003

SEIKI TAKENO
Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan

WENG LIN TANG
Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, 600 South Mathews Ave., Urbana, IL 61801

SANDRA TORRIANI
Department of Science, Technology and Market of Vine and Wine, University of Verona, 37134 Verona, Italy

KELLI TREI
James R. Randall Research Center, Archer Daniels Midland Co., Decatur, IL 62521

ROBERT P. VAN HILLE
Centre for Bioprocess Engineering Research (CeBER), Department of Chemical Engineering, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

JOHAN E. T. VAN HYLCKAMA VLIEG
Danone Research, Gut and Microbiology Platform, R.D. 128, 91767 Palaiseau Cedex, France

BERT VAN LOO
Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, United Kingdom

CARYN VENGADAJELLUM
Centre for Bioprocess Engineering Research (CeBER), Department of Chemical Engineering, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

XIAORU WANG
Natural Products Discovery Group, Department of Chemistry and Biochemistry, 620 Parrington Oval, Room 208, University of Oklahoma, Norman, OK 73019

STEVEN M. WELLS
Verenium Corporation, San Diego, CA 92121

RICHARD C. WILLSON
Dept. of Chemical and Biomolecular Engineering, University of Houston, 4800 Calhoun Rd., Houston, TX 77204-4004

GIDEON M. WOLFAARDT
Dept. of Chemistry and Biology, Ryerson University, 350 Victoria St., Toronto, Ontario, Canada, M5B 2K3

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FENG XU
Novozymes, 1445 Drew Avenue, Davis, CA 95618

XIAOMING YANG
Process and Product Development, Amgen Inc., Thousand Oaks, CA 91320

ZHAOMIN YANG
Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061

AN-PING ZENG
Hamburg University of Technology, Institute of Bioprocess and Biosystems Engineering, Denickestr. 15, 21073 Hamburg, Germany

JINYOU ZHANG
Immunomedics, Inc., 300 American Road, Morris Plains, NJ 07950

NINGYAN ZHANG
Merck Research Laboratories, Merck & Co. Inc., Sumneytown Pike, P.O. Box 4, West Point, PA 19486

HUIMIN ZHAO
Department of Chemical and Biomolecular Engineering and Institute for Genomic Biology, and Departments of Chemistry, Biochemistry, and Bioengineering, University of Illinois at Urbana-Champaign, 600 South Mathews Ave., Urbana, IL 61801

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Author Index

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Heterologous Protein Expression in Yeasts and Filamentous Fungi

NINGYAN ZHANG AND ZHIQIANG AN



11.1. INTRODUCTION

Development of recombinant DNA technologies in the late 1970s laid the foundation for the advancement of various heterologous protein expression systems, including those based on microbial platforms. In the early 1980s, *Escherichia coli* and *Saccharomyces cerevisiae* expression systems were developed and used for heterologous protein expression (47). Many comprehensive reviews of these two expression systems are available (12, 29, 41, 48, 60, 72, 74, 110, 134). Development of other microbial expression systems, such as the methylotrophic yeast *Pichia pastoris* (72) and filamentous fungi (74), followed quickly, and the broad utility of these systems is becoming increasingly apparent.

Heterologous protein expression technologies are widely used in both academic research and industrial applications. In basic research, heterologous gene expression serves as an important tool to prepare proteins and enzymes that are either at limited levels or difficult to purify in their native context. Heterologously expressed proteins and enzymes are used across the entire spectrum of biology. Many enzymes and proteins are of commercial importance, and therefore improved processes for large-scale production of industrial enzymes and proteins are of great interest. Since different enzymes and proteins possess different molecular and biochemical characteristics, different expression systems with specific features are required to meet the growing need for heterologous protein expression at the level of both basic and industrial research.

With the steady advancement in gene expression technology, such as new vectors for gene delivery, improvement in throughput of screening for strain selection, and genetically engineered hosts, production of recombinant proteins has become more efficient with broader applications. In addition, introduction of fusion tags such as hexa-histidine (6× His) and inducible promoters has allowed rapid detection and purification of recombinant proteins and increased expression titers. Since enzymes and proteins have diverse physical and biochemical properties, expression yields vary widely from microgram to low-milligram (37, 57) to multi-gram levels (62). Choosing a suitable expression system for a protein of interest is one of the most critical steps for the development of a high-expression process. Some advantages and drawbacks for commonly used heterologous protein expression systems are listed in Table 1 (138). *E. coli* expression systems provide quick and easy molecular manipulation and, in general, require low-cost growth media. However, the protein expression in *E. coli* cannot be used if foreign proteins require transcriptional and/or posttranslational processing and modifications. By contrast, fungal protein expression systems including yeast and filamentous fungi can provide high expression levels and posttranscriptional and posttranslational processes and modifications of eukaryotic proteins. Similar to *E. coli* protein expression systems, fungal expression systems need low-cost culture media, the technologies needed for scale-up of fungal fermentation processes are well developed, and the organisms are Generally Regarded As Safe (GRAS) (24, 127). In contrast, insect and mammalian expression systems require special culture conditions and relatively high-cost culture media and often have lower expression titers.

When choosing a heterologous protein expression system, the following general criteria should be considered: (i) requirement of translational modification and processing of the protein; (ii) authenticity of the expressed proteins; (iii) protein expression level and scale-up requirement; (iv) localization, e.g., intracellular, membrane-bound, or secreted proteins; (v) cofactor requirement; and (vi) cost, safety, and other regulatory-related issues. Each protein expression system offers a unique set of properties, and for successful production of a heterologous protein, it is often required to tailor a process specifically based on the properties of the protein to be produced and the expression system used.

Many filamentous fungi and yeast species have been used for heterologous protein expression (32, 34, 53, 54, 89, 94, 101, 105, 110, 112, 132, 133). There are numerous reviews addressing foreign protein expressions in each of the fungal protein expression systems (14, 20, 21, 44, 63, 101, 107, 110). Most reviews are focused on one specific system. Furthermore, some address specific aspects of the expression system, such as protein secretion in *S. cerevisiae* (121) and glycosylation of proteins in *P. pastoris* (11). Significant progress has been made in recent years in the production of therapeutic proteins and antibodies using humanized yeast (40). This chapter covers the most commonly used fungal expression systems (*S. cerevisiae*, *P. pastoris*, and *Aspergillus* species), with a focus on vector systems, promoter and leader sequences, posttranslational modifications, and fermentation scalability.

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TABLE 1 Pros and cons for some commonly used heterologous protein expression systems

Expression hosts	Pros	Cons
E. coli	Easy genetic manipulation, rapid growth, simple media requirements and low cost to grow, no human virus carried	Inclusion bodies, no secretion into media, lack of posttranslational process
S. cerevisiae	Low-cost culture and easy to grow, genetic regulation well known, secretion protein, no risk for human virus infection	Overglycosylation at N-linked sites, cell difficult to break, expression level limited
P. pastoris	High-level expression, low cost and easy to grow, genetic regulation well known, secretion protein, no risk for human virus infection	Royalties can be expensive, potential for overglycosylation, cell difficult to break
Filamentous fungi	High level of expression, good secretion system, some level of posttranslational modification, culture medium is inexpensive	No good commercial vectors are available, longer culture time, spores are concerns for health problems, not well documented for production of therapeutic proteins
Insect cells	Easy to grow up to 400 liters, easy to manipulate genetically for baculovirus, can grow in the same-style tanks as mammalian cells	Relatively low expression, serum-free medium is expensive, royalties for the commercially available systems can be costly, few contractors have current Good Manufacturing Practice experience
Mammalian cell lines	Authentic protein expression and proper posttranslational processing and assembly for therapeutic proteins and other mammalian proteins of interest, extensively used, reasonable expression level, endotoxin is not a concern	Medium is expensive, special tank, require CO_2, potential risk of human virus in culture, longer expression time

11.2. *S. CEREVISIAE* FOR HETEROLOGOUS PROTEIN EXPRESSION

S. cerevisiae, a unicellular yeast, is one of the most studied eukaryotic microorganisms. Its genetics, physiology, biochemistry, metabolism, and fermentation are well researched and a wealth of information is readily available. Thousands of genes from *S. cerevisiae* have been characterized (43) and its genome sequence has been determined (33). *S. cerevisiae* has been extensively used as a host for heterologous gene expression of eukaryotic proteins for research and industrial applications (44, 110).

S. cerevisiae is a GRAS organism and has been used for centuries in the brewing and baking industries. Similar to *E. coli*, *S. cerevisiae* requires simple culture media for growth and is easily scaled up for large-volume fermentations. On the other hand, unlike *E. coli*, *S. cerevisiae* is capable of accomplishing many posttranslational modifications, such as proteolytic processing, disulfide bond formation, glycosylation, and other posttranscriptional and translational processing unique to eukaryotic organisms. Many vector systems containing a variety of promoters and auxotrophic or dominant selectable markers have been developed, allowing constitutive or regulated gene expression. Several methods for transformation of foreign DNA into *S. cerevisiae* with high efficiency have also been developed (31, 85). As a result, *S. cerevisiae* is one of the most widely used microbial hosts for heterologous gene expression. However, there are limitations to using *S. cerevisiae* for heterologous gene expression; for example, the specific rate of production can vary from protein to protein, ranging from as low as 0.03 g/kg/h to as high as 4.5 g/kg/h (44). The use of episomal vector systems for heterologous protein expression in *S. cerevisiae* often results in plasmid instability during fermentation, which may lead to a lower growth rate and reduced overall protein yield. Furthermore, since many proteins of therapeutic interest are secreted glycoproteins, the tendency of *S. cerevisiae* to hyperglycosylate proteins not only reduces the efficiency of protein secretion but also may lead to undesired changes in the immunogenic properties or biological activities of the expressed proteins (23, 39, 40, 44, 84).

11.2.1. Vectors

S. cerevisiae expression vectors usually are shuttle plasmids that contain sequences for propagation and selection in both *S. cerevisiae* and *E. coli*. Two types of vectors have been described based on their mode of replication: episomal and integrating vectors (Table 2) (16, 18, 27, 45, 73, 86, 108, 109, 111, 138). Episomal vectors can be characterized according to their copy numbers, mode of replication, and stability. There are three major types of episomal vectors: YRp, YCp, and YEp. YRp-type vectors contain an autonomous replication sequence from the *S. cerevisiae* genome and have an average copy number of 1 to 10 per cell, even though higher copy numbers (up to 100 copies per cell) have been reported (44). These vectors are unstable, and plasmid loss rates can be as high as 10% per generation (44). This instability greatly limits their use in large-scale fermentation processes. YCp-type vectors are derived from the incorporation of *S. cerevisiae* centromeres into YRp plasmids and have improved plasmid stability. However, YCp-type vectors have lower copy numbers, typically at 1 to 2 copies per cell. The most commonly used episomal vectors are of the YEp type derived from the naturally occurring plasmid called 2μ circle in *S. cerevisiae*. These vectors are present at an average of 40 copies per cell and exhibit higher stability than the YRp and YCp vectors (5, 16). Consequently, YEp vectors are the best-developed vectors for heterologous gene expression in *S. cerevisiae*. These vectors are especially useful for controlled expression of toxic proteins. More than 80 compact expression vectors have been developed based

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TABLE 2 Vector systems used for heterologous protein expression in *S. cerevisiae*

Vector	Copy number per cell	Reference
Episomal		
YEp: 2μ-based	25–200	27
YCp: centromere	1–2	18
YRp: replicating	1–20	86
Regulated copy number	3–100	16
Integrating		
YIp	>1	45
rDNA-integrating	100–200	73
Tyδ	<20	111
Transplacement	1	109

on the pRS series of centromeric and 2μ plasmids (26). The GATEWAY vectors (Invitrogen Life Technologies, Carlsbad, CA) incorporated some of these features into their systems in order to provide fast cloning and transfer of genes to different vectors for heterologous expression in most strains of *S. cerevisiae*. Other 2μ-based expression vectors available from commercial sources include YEpFLAG-1 from Sigma (St. Louis, MO), YES vectors from Invitrogen, and pESC vectors from Stratagene (La Jolla, CA).

Integrative vectors contain selectable *S. cerevisiae* genes and lack sequences for autonomous replication. These vectors are highly stable but usually present at low copy numbers. They include the YIp-type vectors, and their integration can be directed by homologous recombination between sequences carried on the plasmid and its homologous counterpart in the *S. cerevisiae* genome. Some integration vector systems use repetitive elements such as delta sequences, Ty elements, or tRNA genes for heterologous gene integration (119). A system for multiple-site integration of a heterologous gene into the ribosomal DNA (rDNA) 9.1-kb segment has also been developed (73). Stability of the genome-integrated expression cassette can be maintained under selection. However, a decrease of copy numbers of the integrated expression cassette was observed under nonselective culture conditions (44).

11.2.2. Promoters

An array of *S. cerevisiae* promoters has been used for heterologous gene expression. There are constitutive promoters derived from genes such as *CYC1* (cytochrome C oxidase), *TEF2* (translation elongation factor), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), as well as regulated promoters such as *GAL1* (galactokinase/galactose epimerase 1), *ADH2* (alcohol dehydrogenase 2), *CUP1* (metallothionein), and *PHO5* (acid phosphatase) (6, 10, 15, 46, 51, 55, 56, 58, 65, 66, 80, 97, 98, 124). It is often advantageous for improving expression titer to couple the protein production phase with cell growth phases if there is no toxicity issue for the protein of interest. Some inducible promoter systems that work well at the shake flask scale, such as temperature-regulated promoters, do not always function well in large-scale fermentation due to operational restraints (44). Hybrid promoters such as *GAP/GAL* and *GAL10/CYC1* have also been reported (6). Several reviews on promoters and their applications in *S. cerevisiae* are available (108, 128, 138).

11.2.3. Transcriptional and Translational Regulation

In order to fully utilize the transcriptional, translational, and posttranslational regulatory machinery and to get efficient heterologous gene expression in *S. cerevisiae*, it is very important to match the expressed protein with the proper expression vector system. There have been many studies and extensive reviews on these aspects (22, 44, 82, 123). Generally, the use of strong promoters and robust transcriptional terminators of *S. cerevisiae* is essential for maximal expression since most prokaryotic or higher eukaryotic transcription terminators are not active in *S. cerevisiae*. More importantly, mRNA levels are controlled by the rate of transcription initiation and the transcript turnover rate (mRNA stability). In addition to upstream regulatory elements, downstream activation sequences are also required for maximum transcription initiation in *S. cerevisiae* (123). Expression levels of some foreign genes with high AT content (>60%) in *S. cerevisiae* can be low or absent due to incomplete transcript elongation (108). Transcript-destabilizing sequence elements have been reported in the 5′ nontranslated regions, coding regions, and 3′ nontranslated regions of different mRNAs (22). Therefore, if the titer is low due to the instability of mRNA, it may be necessary to use a stronger promoter, to delete the transcript-destabilizing sequence elements, and to introduce alternative codons. It has been shown that secondary structure of mRNA is one of the most important factors affecting the rate of initiation and translational elongation (123). For secreted proteins, posttranslational processes such as proteolytic cleavage, N-terminal modification, and glycosylation are critical to both proper protein folding and high protein yields. Consequently, it is worthwhile to evaluate the effect of the posttranslational steps when strong promoters and different strains fail to produce desired product yields.

S. cerevisiae has been used for expression of many eukaryotic proteins in both intracellular and secreted forms. For production of secreted proteins, secretion signals can come either from native protein signal sequences or from *S. cerevisiae* secretion protein signal sequences. Due to the limited studies available in the literature on the effects of different signal peptides on the yield of heterologous protein expression in *S. cerevisiae* (120), it is a good starting point to include the signal sequence of the foreign protein in the initial test of expression cassettes. Alternatively, *S. cerevisiae* signal sequences from invertase (SUC2, 19 amino acids), acid phosphatase (PHO5, 17 amino acids), and the most widely used α-factor pheromone (MFα1, 20 amino acids) can be incorporated into the recombinant construct(s).

An important step among posttranslational modification processes is glycosylation. Heterologous glycoproteins expressed in *S. cerevisiae* are glycosylated at both N-linked and O-linked sites (64). Little is known about O-linked glycosylation, but more information is available on the process of N glycosylation. In *S. cerevisiae*, a core sugar moiety consisting of two *N*-acetylglucosamines (GlcNAc), nine mannoses, and three glucoses is added to the N-amide of asparagine at the Asn-X-Ser/Thr sequence in the endoplasmic reticulum (ER) in the early process of N glycosylation (67). Three glucose residues and one mannose residue are subsequently removed in the early Golgi apparatus. It is in the Golgi apparatus where further modification takes place that results in major differences between *S. cerevisiae* and higher eukaryotes in oligosaccharide structures. In higher eukaryotes, additional mannose residues are removed and several other sugars such as galactose, sialic

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acid, and fucose are added (39, 40). *S. cerevisiae* maintains the core mannose scaffold and is often extended with a large number of additional mannose residues. This often results in a hyperglycosylated outer chain containing more than 50 mannose residues with many branch chains. Many examples of hyperglycosylation of heterologous proteins in *S. cerevisiae* have been reported (23, 68, 84). Since specific glycosylation patterns are important for the immune response in mammalian systems, wild-type *S. cerevisiae* is not a good system for the production of therapeutic proteins such as monoclonal antibodies that have complex sugar structures.

11.2.4. Industrial Protein Production in *S. cerevisiae*

Many heterologous proteins have been produced in *S. cerevisiae* and large-scale fermentation processes have been developed (43, 108). Nevertheless, most heterologous protein production in *S. cerevisiae* remains in shake flask cell cultures. The first therapeutic recombinant protein expressed in *S. cerevisiae* was human alpha interferon (47). Since then, many other therapeutic proteins have been expressed in *S. cerevisiae*. These include the hepatitis B surface antigen (126), human insulin (122), the human papillomavirus (HPV) vaccine Gardasil™ (93, 135), and many others. In the case of Gardasil, the four vaccine components were expressed in *S. cerevisiae*, which were transformed by pGAL110-based yeast expression vectors, each containing the gene of interest coding for HPV L1 types 6, 11, 16, or 18 (75, 104). An excellent review by Vasavada (128) details a comprehensive list of foreign proteins expressed in *S. cerevisiae* at small and large industrial scale. Various strategies have been reported to improve the expression levels of different heterologous proteins in *S. cerevisiae*, such as screening of various host strains with different expression vectors, altering host genetics by molecular engineering, and optimization of fermentation conditions (83, 115, 128). Since there are no defined conditions that can be used for all protein production, it is a general rule of thumb to examine different parameters to develop an optimized process for heterologous protein expression in *S. cerevisiae*.

11.3. *P. PASTORIS* FOR HETEROLOGOUS PROTEIN EXPRESSION

Pichia expression systems have been widely used for expression of heterologous proteins from a diverse array of organisms, including bacteria, fungi, protists, plants, invertebrates, nonhuman vertebrates, and humans (72). Similar to *S. cerevisiae*, protein expression systems in *Pichia* have the advantages of well-established, cost-effective fermentation technology and comprehensive understanding of the host molecular genetics. *P. pastoris* is a methylotrophic yeast and has strong preference for respiratory growth (72). Cell cultures can reach extremely high density, with dry weight mass reaching as high as 100 g/liter (44). Most of the *Pichia*-based protein expression systems use a tightly controlled and highly inducible promoter derived from the alcohol oxidase 1 gene (*AOX1*). Under methanol-induced conditions, a single protein such as alcohol oxidase can reach as high as 30% of the total cell proteins (44, 72). Similar methods used for the molecular genetic manipulation for *S. cerevisiae* can be applied to *P. pastoris*. Extensive studies have been published on the use of *Pichia* systems in heterologous protein expression. A recent review article by Cregg et al. (14, 20) listed 241 individual studies of heterologous proteins expressed in *P. pastoris*, more than 80 of which were human proteins. Expression levels for heterologous proteins differ greatly, ranging from microgram-per-liter to gram-per-liter levels. For example, a hydroxynitrile lyase from a tropical rubber plant (*Hevea brasiliensis*) was expressed at levels as high as 22 g/liter (42). There are numerous reports on heterologous protein expression in *P. pastoris* (9, 91, 94, 107, 113, 114, 130, 138).

11.3.1. Expression Vectors and Host Strains

A fully developed *P. pastoris* expression system is commercially available (Invitrogen). The *Pichia* vectors can be grouped into two categories based on their promoters used: (i) inducible promoters derived from the *AOX1* gene or the formaldehyde dehydrogenase gene (*FLD1*) (117), and (ii) a strong constitutive promoter derived from the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene (*pGAP*). The methanol-inducible *AOX1* promoter provides tighter control of gene expression. The *FLD1* promoter can be induced by either methanol as the sole carbon source or certain methylated amines such as methylamine as the sole nitrogen source. Alternatively, the constitutive *GAP* promoter does not require cultures to be shifted from one carbon source to another for the induction of heterologous gene expression. However, if the foreign proteins to be expressed are toxic to the host cells, constitutive expression of the enzyme or proteins may cause inhibition of cell growth or result in loss of the expression vector from the cell.

There are no stable episomal expression vectors available in *P. pastoris*, and the expression genes need to be integrated into the genome to obtain stable expression strains. The simplest way of integration is single crossover homologous recombination by digesting the vector at a unique site with a restriction enzyme within either the marker gene (e.g., *HIS4*) or the promoter sequences (e.g., *AOX1*) (117). In the case of *HIS4* as the selectable marker, transformants of auxotrophic mutant strain (*HIS4*) can be selected for His^+ cell growth. Alternatively, expression strains can be generated with *AOX1* flanking sequences at both the 5′ and 3′ terminals by cutting the vector in the *AOX1* sequence region. The flanking *AOX1* sequences at both ends can stimulate gene replacement events at the *AOX1* site in the *Pichia* genome, and this can result in a gene replacement integration. Heterologous proteins expressed in *P. pastoris* can be retained inside cells (intracellular expression) or secreted into medium depending on signal sequences in the expression vector systems. Commercial vectors for secreted protein expression often carry a leader sequence from MFα1 (mating factor) of *S. cerevisiae* as a secretion signal (117). Other leader sequences, such as the alkaline phosphatase (PHO) signal sequence from *P. pastoris* and some native protein leader sequences, have also been used for secretion of foreign proteins in *Pichia* (79). To facilitate protein detection and purification, most vectors have 6× His or *c-myc* tag sequences fused at the C-terminal or N-terminal end.

A variety of selection markers are available for *Pichia* protein expression. Some can be used for selecting multicopy gene integration, such as the drug resistance markers zeocin and blasticidin, while others provide auxotrophic selection such as $his4^-$ mutant strains. Commercially available *Pichia* host strains for protein expression include X-33, GS115, SMD1168, and KM71. Additional host strains for

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P. pastoris heterologous protein expression are reviewed by Cregg et al. (20).

11.3.2. Protein Folding and Glycosylation in *P. pastoris*

P. pastoris, similar to *S. cerevisiae* and other fungi species, can perform protein posttranslational modifications, such as cleavage of signal sequences for secreted proteins, N- and O-linked glycosylation, and formation of disulfide bonds (71, 72). It has been well documented that overexpression of heterologous proteins in *P. pastoris* can have a deleterious effect on its secretion pathway. Protein folding in the ER and Golgi is often the rate-limiting step for heterologous protein expression. Overloading the secretion pathway can lead to accumulation of partial or misfolded heterologous proteins in cells. Existence of high levels of unfolded or misfolded proteins in cells may induce a stress reaction termed the "unfolded protein response" (UPR), which triggers the expression of folding chaperones and enzymes in addition to other physiological responses. Both heat shock protein 70-type chaperone (BiP) and a protein disulfide isomerase (Pdi1) were reported to be induced when cells face UPR in *Pichia* (44, 72). BiP and Pdi1 showed synergistic effects on improving secretory capacity (136).

Glycosylation is one of the most important posttranslational modifications for heterologous protein expression, since it not only affects proper folding and secretion of the protein but also has a direct impact on the biological activities of proteins. Both N- and O-linked glycosylation have been reported using *Pichia* systems for heterologous protein expression (20). The consensus sequence for N-linked glycosylation in *Pichia* is the same as that in *S. cerevisiae* (Asn-X-Thr/Ser), in which Ser or Thr provides a hydroxyl group for glycosylation. Similar to *S. cerevisiae* and other fungal organisms, *Pichia* transfers a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$, to an amino acid (Asn) in the ER. This oligosaccharide core is further modified in the Golgi. Unlike hyperglycosylation (>50 Man) occurring in *S. cerevisiae*, the most commonly found N glycosylation structure in *P. pastoris* is $Man_{14}GlcNAc_2$. There has been no α-1,3-mannosyltransferase activity detected in *P. pastoris*; only α-1,6 and α-1,2 linkages were found in glycosylated proteins in *Pichia* (11). The O-linked oligosaccharides in *Pichia* are also reported, but a consensus sequence for O-linked glycosylation is not available. The O-linked oligosaccharides in *P. pastoris* are generally short (<5 residues) and contain only α-1,2-linked mannose units. Phosphorylation of oligosaccharide units has been found in *P. pastoris* expression systems, but the exact sites of phosphate linkage on saccharides are unknown (72). In order to produce antibodies for therapeutic use, extensive glycoengineering efforts have been carried out to make humanlike glycoforms in *Pichia* (39, 40). Fully humanized sialylated antibody was produced successfully in a humanized *Pichia* strain (39). It is noteworthy that unlike antibodies produced in mammalian cells, antibodies produced in humanized *Pichia* do not contain fucose in the core carbohydrate structures and the afucosylated antibodies showed increased binding affinity to Fcγ receptor IIIA, which in turn results in higher antibody-dependent cellular cytotoxicity (70).

11.3.3. Heterologous Protein Production in *Pichia*

Expression levels of heterologous proteins in *P. pastoris* vary greatly depending on copy numbers of the foreign gene integrated and the stability of both mRNA and protein. Since expression levels are correlated with gene copy number, it is important to achieve high copy numbers of gene integration. To obtain multiple copies of the target gene integrated in *Pichia*, one can screen large numbers of transformants in a 96-well plate format, applying high concentrations of the selection agents (e.g., zeocin and blasticidin), or construct multiple copies of the gene in tandem repeats in vitro.

In addition to the high-copy-number transformants, optimization of the expression process is vital to achieve high-level expression of heterologous proteins in *Pichia*. Although some proteins can be expressed well in flask cultures, it is common that expression levels are greatly enhanced in a controlled environment. Cultures in fermentor vessels offer several advantages over flask cultures, such as control of pH and dissolved oxygen levels, and continuous feeding of carbon and other components during cell growth and protein expression (49, 72). Since *P. pastoris* cells have a preference for respiratory growth, cultures can be grown to extremely high cell densities (up to 500 absorbance units at 600 nm) (20, 21). This is particularly important for secreted proteins since the concentration of the expressed proteins is often proportional to the concentration of cells in culture. Common defined media for *P. pastoris* protein expression are composed of glucose and glycerol as the carbon source and Casamino Acids and/or ammonium salts as the nitrogen source in addition to biotin and trace elements. The *Pichia* fermentation for heterologous protein expression can be separated into two phases. The first phase is that of growth using glycerol as a carbon source, and at this stage the *AOX1* promoter is fully repressed. After depletion of glycerol, a transitional phase may be added to feed more glycerol at a growth-limiting rate. The second phase of feeding methanol starts induction of the *AOX1* promoter and heterologous gene expression. Detailed protocols for fermentation of *P. pastoris* can be found in various articles (30, 50, 72, 90, 96).

Similar to other heterologous protein expression systems, proteolytic hydrolysis often causes instability of foreign proteins in *Pichia*. In addition to use of proteinase-deficient strains, several fermentation strategies can be applied to control proteolysis of foreign proteins. Some common approaches that have been used successfully to reduce hydrolysis of heterologous proteins in *Pichia* include (i) adding amino acid-rich supplements such as peptone or Casamino Acids; (ii) culturing at more acidic pH values (*Pichia* cultures grow well between pH 3 and 7); (iii) using a protease (*PEP4*, vacuolar protease gene)-deficient strain; and (iv) controlling culture temperature (44, 72). Another limitation of heterologous protein expression in *Pichia* is inefficient secretion of some mammalian proteins. In these cases, alternative protein secretion sequences can be tested first, followed by the consideration of more extensive molecular engineering approaches to improve protein chaperone and folding machinery.

11.4. HETEROLOGOUS PROTEIN EXPRESSION IN FILAMENTOUS FUNGI

Filamentous fungi, most notably *Aspergillus* species, can produce high levels of proteins and are capable of secreting proteins into the media. Many industrial enzymes, such as amylase, invertase, pectinase, and cellulases, that are used widely in the food industry for production of high-fructose corn syrup, fruit juice, and wine are produced in *Aspergillus*

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species. The most well-known species for producing commercial enzymes include *A. oryzae* and *A. niger*; both have been given GRAS status by the U.S. FDA (116) for production of enzymes and proteins (35). The GRAS status makes them more attractive for use in the production of enzymes and proteins for the food industry and for pharmaceutical products such as therapeutic proteins and antibodies (29, 131). However, in comparison with *S. cerevisiae* and *P. pastoris*, filamentous fungi are less studied for heterologous protein production with respect to their transcription and translation control, mRNA stability, glycosylation, and regulation of protein secretion. With growing demand for the production of therapeutic proteins including antibodies, there is renewed interest in developing cost-effective fungal expression systems including yeast and *Aspergillus* species for heterologous protein production. In recent years, many advances have been made in the use of filamentous fungi for protein production (74, 87, 129).

11.4.1. Host Strains and Selection Markers for Transformation

Unlike *Pichia* and *Saccharomyces*, there are no commercially available protein expression systems for *Aspergillus*. However, there are several Web-accessible organizations such as the ATCC (American Type Culture Collection), the FGSC (Fungal Genetics Stock Center), the NRRL (Agricultural Research Service Culture Collection), and the BCCP (Belgium Culture Collection) from which the necessary tools for heterologous protein expression in filamentous fungi can be obtained.

For heterologous protein expression in *Aspergillus* species, the first step is to select a suitable host strain. The expression host must be either auxotrophic or antibiotic susceptible so that it can be used for efficient transformation and selection of transformants. The most genetically accessible strain is *A. nidulans*, but *A. niger*, *A. awamori*, and *A. oryzae* are more extensively used because of their capacity for high-level expression of commercial enzymes. *A. niger* can efficiently secrete many enzymes including food-related enzymes. *A. oryzae* has become more amenable to molecular analysis, and current research is focused on the identification of strong promoters to improve expression of industrially valuable enzymes. Due to the limited number of studies on *Aspergillus* strains and patent restrictions on the use of some strains and markers, more studies are warranted to fully utilize the potential of *Aspergillus* host strains for heterologous gene expression.

Transformation of host strains can result in chromosomal integration of plasmids, either through a random mechanism or through site-directed integration. Markers commonly used for transformation of auxotrophic strains are listed in Table 3 (7, 13, 59, 81, 100–102, 125, 137, 138). The *pyrG* gene (uracil/uridine auxotrophs) from *A. niger* is a common marker found in many plasmid vectors. The *A. nidulans argB* gene is also used extensively for efficient transformation of suitable auxotrophic host strains. For example, Buxton et al. (13) described the complementation of an auxotrophic *A. niger* strain with the *A. nidulans argB* gene. More recently, there was a report on the complete disruption of the *argB* gene on chromosome I in *A. niger* (69). The *A. niger argB* deletion strain can be complemented by the *argB* genes from both *A. niger* and *A. nidulans* and can serve as a useful host strain for heterologous protein expression in *A. niger*.

Although plasmids can be readily integrated into the chromosome (stable and multicopy integration) during transformation of *Aspergillus* species, it is desirable to have a replicative plasmid for heterologous protein expression in filamentous fungi. Sequences have been isolated that allow one to perform replicative transformation in *A. nidulans* (2). Initial work with linear plasmids containing human telomeric elements was encouraging, but later these were shown to be unstable (3). To date, a synthetic *Aspergillus* replicative plasmid of wide utility is not yet available (74).

11.4.2. Expression Vectors and Promoters

Promoters commonly used for heterologous protein expression in *Aspergillus* species are listed in Table 4 (17, 25, 35, 38, 61, 99, 103). Constitutive promoters include the *trpC* and the *gpdA* promoters from *A. nidulans*. Other frequently used promoters are the starch-inducible promoters, α-amylase and the glucoamylase promoters from *A. oryzae* and *A. niger*. The glucoamylase promoter responds to maltose as a carbon source and is repressed by glucose. More recently the *TEF1-α* promoter of *A. oryzae* has been studied for use as a strong constitutive promoter (61).

TABLE 3 Selectable markers used for heterologous gene expression in *Aspergillus* species

Gene markers	Enzyme	Cellular function	Gene host	Reference
Auxotrophic				
argB	Ornithine carbamoyltransferase	Arginine biosynthesis	*A. nidulans*	13
pyr4	Orotidine 5′-phosphate decarboxylase	Uracil biosynthesis	*Neurospora crassa*	7
pyrG	Orotidine 5′-phosphate decarboxylase	Uracil biosynthesis	*A. niger*	81
trpC	Phosphoribosylanthranilate isomerase	Tryptophan biosynthesis	*A. nidulans*	137
amdS	Acetamidase	Acetamide or acrylamide assimilation, N_2 source	*A. nidulans*	59
niaD	Nitrate reductase	Nitrate assimilation, N_2 source	*A. niger*	125
Dominant				
hph (HmB^r)	Hygromycin B phosphotransferase		*E. coli*	100
phleo^r	*E. coli* transposon Tn5 phleo^r DNA			102

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TABLE 4 Promoters used in heterologous gene expression in *Aspergillus* species

Gene	Enzyme	Species	Reference(s)
Strong inducible			
alcA	Alcohol dehydrogenase 1 (ADH1) (ethanol inducible)	*A. nidulans*	25
α-Amylase	Starch inducible	*A. oryzae*	17
glaA	Glucoamylase (starch and maltose inducible)	*A. nidulans*	36
Constitutive			
gpdA	Glyceraldehyde-3-phosphate dehydrogenase	*A. nidulans*	99, 103
trpC	Phosphoribosylanthranilate isomerase	*A. nidulans*	38
TEF1-α	Translation elongation factor 1-α	*A. oryzae*	61

Another suitable promoter for the expression of heterologous proteins in filamentous fungi is the ethanol-regulated promoter *alcA* for alcohol dehydrogenase from *A. nidulans*. Genes for ethanol utilization in *A. nidulans* are efficiently transcribed. The *alcA* promoter is tightly regulated through *alcR*, a *trans*-activating gene, and is subject to repression by the major carbon catabolite repressor CreA (25). In a recent report, expression of antisense *creA* in *A. nidulans* increased levels of expression for glucose-repressible enzymes in the presence of glucose (8). The derepression effect by the *creA* antisense DNA did not alter cell morphology and growth parameters, as observed in a *creA* null mutant. The *alcR-alcA* regulatory system from *A. nidulans* has been transformed into *A. niger*, which does not possess an inducible *alc* system (88). The *alcR-alcA*-transformed strain of *A. niger* showed similar levels of heterologous expression of the reporter enzyme β-glucuronidase, as in *A. nidulans*.

Ishida et al. (52) reported that the tyrosinase gene promoter (*melO*) was four times stronger for the heterologous expression of β-glucuronidase from *E. coli* in a submerged culture as compared with other gene promoters, such as *amyB*, *glaA*, and *agdA*, previously used for heterologous gene expression in *A. oryzae*. The *melO* promoter was used to produce the *glaB*-type glucoamylase in a submerged culture of *A. oryzae* and achieved maximum yield of 3.3 g/liter and 99% purity. This work establishes the *melO* promoter for high-level expression and high-purity production of heterologous proteins in *A. oryzae*.

11.4.3. Posttranslational Regulations

Large numbers of mammalian enzymes and proteins have been expressed through genetic engineering; however, secretion of heterologous proteins is often much less efficient than the secretion of native fungal proteins. During the last 10 years, the production levels of heterologous proteins in *Aspergillus* have been improved by fusing the protein of interest to the 3′ end of a highly expressed gene encoding a protein that is efficiently secreted from the host. The gene fusion or carrier protein strategy has been used with the glucoamylase gene from *A. niger* or *A. awamori*. Increases of 5- to 1,000-fold in protein production levels have been observed (87). Gouka et al. (36) extensively reviewed the production levels of different proteins fused to the glucoamylase carrier gene. They also performed detailed experiments on the production levels of various proteins with carrier fusions to the glucoamylase protein in isogenic, single-copy strains of *A. awamori*. The glucoamylase gene can be divided into three distinct domains: the catalytic N-terminal domain, the C-terminal starch-binding domain, and a flexible linker region that separates the two domains. The C-terminal starch-binding domain can be replaced by a heterologous protein. The glucoamylase carrier proteins are believed to improve translocation in the ER, to enhance proper protein folding, and possibly to afford protection against proteolysis. In the secretory pathway, the carrier protein can be cleaved by a KEX2-like protease at a specific proteolytic cleavage site separating the glucoamylase carrier and the protein of interest. The amino acid sequence (NVISKR) of the cleavage site is derived from the propeptide of glucoamylase. The carrier approach appears to be the first choice in attempts to produce secreted proteins in *Aspergillus* species. There are many reports on the use of carriers for heterologous protein secretion. A recent study reported a successful secretion of laccase (*lac*1) (an industrially useful enzyme for the oxidation of *p*-diphenols) from *Pycnoporus cinnabarinus* that used either the native laccase signal sequence or the *A. niger* glucoamylase pre-pro-sequence as a carrier for the heterologous enzyme expression in *A. niger* (106). The fusion of the glucoamylase pre-pro-sequence increased expression levels 80-fold in comparison to the levels observed with the laccase signal peptide sequence. Another advantage of using the glucoamylase pre-pro-sequence as a carrier for laccase in *A. niger* is the production of the 70-kDa protein, which is similar to the molecular mass of the native protein (106). However, it was reported that laccase expressed in *P. pastoris* had an estimated 110-kDa molecular mass due to hyperglycosylation in the yeast (76, 77, 106). Human interleukin-6 (IL-6) secretion was also improved using the native glucoamylase as a carrier when expressed in *A. niger* (74). It has been reported (4) that the UPR, i.e., the deliberate upregulation of "foldases" and chaperones, can also improve the secretion of heterologous proteins.

Efficient heterologous protein production in fungi cannot always be achieved by alleviating the limitations of transcription. Posttranslational regulations such as glycosylation, protein folding, and secretion often pose challenges for successful expression of heterologous proteins in *Aspergillus* species. Another major limiting factor in protein production in filamentous fungi is proteolytic degradation by high levels of intracellular and extracellular proteases. *A. niger* has four predominant extracellular proteases, two aspartyl proteases (PEPA and PEPB) and two serine carboxypeptidases (PEPF and PEPG). In *A. nidulans*, neutral and alkaline proteases often are the dominant ones affecting the

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yields of heterologous proteins. Efforts to eliminate specific protease activities through mutagenesis or gene disruption have improved protein production levels. Van den Hombergh et al. (127) reviewed fungal proteases and described the development of a set of tester strains of *A. niger* that may be used to identify the appropriate genetic background for a particular protein.

Posttranslational modifications such as N glycosylation are particularly important for many pharmaceutically relevant proteins because of their impact on biological activities (19). High mannose-type glycosylation is observed in filamentous fungi, but it is much less extensive than that seen in proteins produced in *Saccharomyces* and *Pichia* species. Maras et al. (78) have nicely reviewed the fungal glycosylation pathway and approaches and compared the carbohydrate structures with proteins produced in mammalian cells.

Successful expression of *Drosophila melanogaster* xanthine dehydrogenase in *A. nidulans* is a recent example of proper posttranslational modification of a heterologous enzyme in filamentous fungi (1). In comparison to the baculovirus expression system, which produced only about 10% of active xanthine dehydrogenase enzyme due to incomplete incorporation of the cofactor molybdenum and two iron-sulfur clusters, *A. nidulans* produced 40% of active enzyme with relatively more complete cofactor incorporation.

11.4.4. Scale-Up of Fermentation and Protein Production in *Aspergillus*

In addition to the molecular genetic approaches, a combination of host strain selection, strain development, and production process development including media optimization have resulted in commercially attractive protein yields in *Aspergillus* protein expression systems (101). For example, IL-6 gene carrier fusion in *A. nidulans* in combination with host strain development and growth optimization resulted in a 200-fold increase in production of active IL-6 (101). A combination of gene dosage and growth condition optimization for the improvement of protein expression in *A. oryzae* resulted in a high production titer of phospholipase A_1 (PLA1) at the industrial scale (118). Transformants with 15 to 20 copies of the *PLA1* gene were integrated in the chromosome of *A. oryzae* and produced 60- to 7,500-fold higher activity than the native expression level. In addition, improvement of the mycelial morphology from a pellet form to a filamentous form improved production of PLA1 in scaled-up fermentations and resulted in production of PLA1 at 1.8 g/liter consistently. This is consistent with the observation that improvements in oxygen transfer and broth rheology obtained with the pellet form of growth often resulted in increased protein production.

It has been recognized that fungal morphology plays an important role in determining process productivity in mycelial fermentations. This has led to investigations into alternative ways to conduct fermentations to optimize production yields. Papagianni et al. investigated the effect of cell immobilization on protein production and secretion (92). They reported on cells of *A. niger* that were immobilized by attachment to metal surfaces or by spore entrapment, and subsequently grown on Celite beads. They noted that cell attachment on metal surfaces decreased secretion of proteases and increased glucoamylase production. It was observed that growth on Celite beads in shake flasks reduced the secreted proteases by half and almost doubled the specific activity of glucoamylase. In bioreactor cultures, conditions can be better controlled than in flask culture and effects of culture conditions on protein yields are much greater.

11.5. FUTURE PROSPECTS

The demand for production of a variety of proteins and enzymes in heterologous hosts for research and industrial applications is growing continuously. It is of great interest to develop and optimize diverse host systems for heterologous protein expression to meet the growing demand. Fungal expression systems offer many advantages over other expression systems and have been widely used for production of heterologous proteins from many organisms. Discovery of new promoters and development of new vectors will provide more choices for different protein expression needs. Greater understanding of transcriptional control, mRNA stability, glycosylation, protein folding, and other posttranslational processes will allow us to overcome the difficulties associated with heterologous protein expression in fungi, such as hyperglycosylation in yeasts and poor secretion associated with some mammalian proteins. It has been documented that the use of protease-deficient strains can increase the yield of recombinant protein production (28, 95) and that the use of specific mutants may eliminate hyperglycosylation problems with some proteins expressed in *Saccharomyces* (115). The exponential increase in fungal genomics and proteomics in recent years will no doubt fuel the advancement of filamentous fungi as heterologous protein expression hosts (74). Collectively, fungal protein expression systems have been and will continue to serve as a choice for heterologous protein production in small or large quantities.

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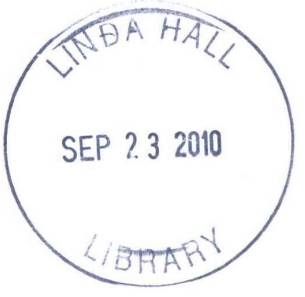
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Research Article

Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast *Pichia pastoris*

Balla Venkata Kranthi, Hunsur Rajendra Vinod Kumar and Pundi N. Rangarajan*
Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

*Correspondence to:
Pundi N. Rangarajan,
Department of Biochemistry,
Indian Institute of Science,
Bangalore 560012, India.
E-mail: pnr@biochem.iisc.ernet.in

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Abstract

Expression of genes involved in methanol metabolism of *Pichia pastoris* is regulated by Mxr1p, a zinc finger transcription factor. In this study, we studied the target gene specificity of Mxr1p by examining its ability to bind to promoters of genes encoding dihydroxyacetone synthase (*DHAS*) and peroxin 8 (*PEX8*), since methanol-inducible expression of these genes is abrogated in *mxr1*-null mutant strains of *P. pastoris*. Different regions of *DHAS* and *PEX8* promoter were isolated from *P. pastoris* genomic DNA and their ability to bind to a recombinant Mxr1p protein containing the N-terminal 150 amino acids, including the zinc finger DNA-binding domain, was examined. These studies reveal that Mxr1p specifically binds to promoter regions containing multiple 5′-CYCC-3′ sequences, although all DNA sequences containing the 5′-CYCC-3′ motif do not qualify as Mxr1p-binding sites. Key DNA-binding determinants are present outside 5′-CYCC-3′ motif and Mxr1p preferably binds to DNA sequences containing 5′-CYCCNY-3′ than those containing 5′-CYCCNR-3′ sequences. This study provides new insights into the molecular determinants of target gene specificity of Mxr1p, and the methodology described here can be used for mapping Mxr1p-binding sites in other methanol-inducible promoters of *P. pastoris*.
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Keywords: ***Pichia pastoris*; Mxr1p; methanol metabolism; alcohol oxidase; dihydroxyacetone synthase; peroxin 8**

Introduction

Methanol expression regulator 1 (Mxr1p) is a novel zinc finger protein essential for the methanol-inducible expression of genes involved in methanol utilization pathway of *Pichia pastoris* (Lin-Cereghino *et al.*, 2006; Hartner *et al.*, 2008). The amino-terminal zinc finger motif of Mxr1p shares 70% identity with the zinc finger DNA-binding domain of *Saccharomyces cerevisiae* Adr1p (alcohol dehydrogenase II synthesis regulator), a transcription factor regulating the expression of genes involved in the metabolism of glycerol, ethanol and oleic acid (Lin-Cereghino *et al.*, 2006). Mxr1p is the *P. pastoris* homologue of *Saccharomyces cerevsiae* Adr1p and it was suggested that Mxr1p and Adr1p may bind similar DNA sequences (Lin-Cereghino *et al.*, 2006). The sequence 5′-CYCCRD(N4-36)HYGGRG-3′ is the consensus sequence for Adr1p (Shuster *et al.*, 1986; Eisen *et al.*, 1988; Cheng *et al.*, 1994; Cheng and Young, 1995) and DNA sequences similar to *S. cerevisiae* Adr1p-binding sites are present in the promoter of the gene encoding alcohol oxidase I (*AOX1*), the first enzyme involved in methanol metabolism (Lin-Cereghino *et al.*, 2006; Hartner *et al.*, 2008). Deletion of the CYCC motif of the putative Adr1p-binding site of *AOX1* promoter was

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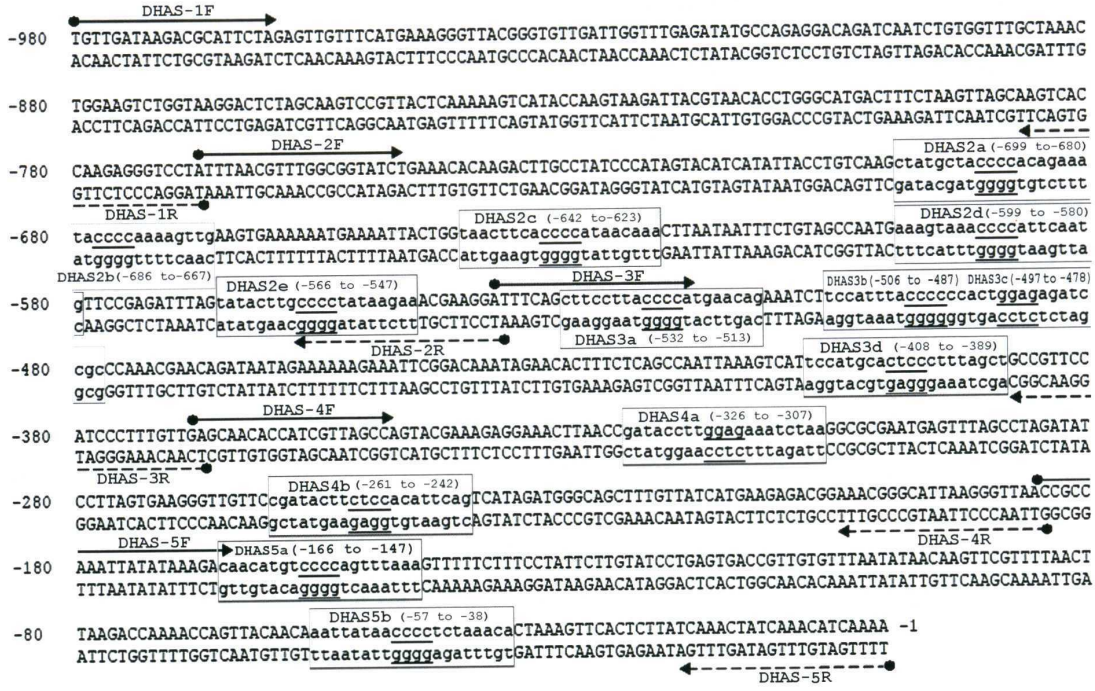


Figure 1. Nucleotide sequence of *DHAS* promoter and strategy for the PCR amplification of promoter DNA fragments. The forward (F) and reverse (R) primers used in PCR reactions to obtain amplicons encompassing five different promoter regions (DHAS1-5) are indicated by unbroken and broken arrows, respectively. Oligonucleotides (20 bp) containing the core 5′-CYCC/GGRG-3′ sequence (underlined) used in EMSA studies (Figure 3b) are boxed

shown to result in 70% decrease in *AOXI* gene expression, indicating a key role for this sequence in *AOXI* gene regulation (Hartner *et al.*, 2008). In addition to *AOXI*, expression of a number of other genes involved in the methanol utilization pathway, such as those encoding dihydroxyacetone synthase (DHAS), formaldehyde dehydrogenase (FLD), peroxins 5, 8, and 14 (PEX5, PEX8, PEX14), is also affected in *mxr1*-null mutant strains (Lin-Cereghino *et al.*, 2006). Whether methanol-inducible expression of these genes is mediated by the direct binding of Mxr1p to CYCC motifs present in their promoters is not known.

When *P. pastoris* cells are grown in methanol, expression of enzymes of methanol metabolism, as well as proteins involved in peroxisome biogenesis (peroxins), is induced (Cregg *et al.*, 1989; Lin-Cereghino *et al.*, 2000; Hartner *et al.*, 2006). During methanol metabolism, methanol is first converted to formaldehyde by AOX, which is further oxidized by FLD and formate dehydrogenase (FDH) to generate energy. In another pathway, formaldehyde is condensed with xylulose 5-phosphate by DHAS to generate two three-carbon molecules that enter a cyclic pathway leading to the regeneration of xylulose 5-phosphate, as well as synthesis of one net molecule of glyceraldehyde 3-phosphate for every three runs of this cycle. The reactions catalysed by AOX and DHAS occur in peroxisomes and therefore, peroxisomes are essential for growth of *P. pastoris* in methanol (Cregg *et al.*, 1989; Lin-Cereghino *et al.*, 2000; Hartner *et al.*, 2006). When the carbon source is switched from glucose to methanol, in addition to *AOX, FLD* and *DHAS*, expression of a number of *PEX* genes, many of which encode components of peroxisome import machinery, is also induced. In a recent study (Kranthi *et al.*, 2009), we have identified six Mxr1p-binding sites in the *AOXI* promoter, each of which consists of a core 5′-CYCC-3′ motif. In this study, we have examined

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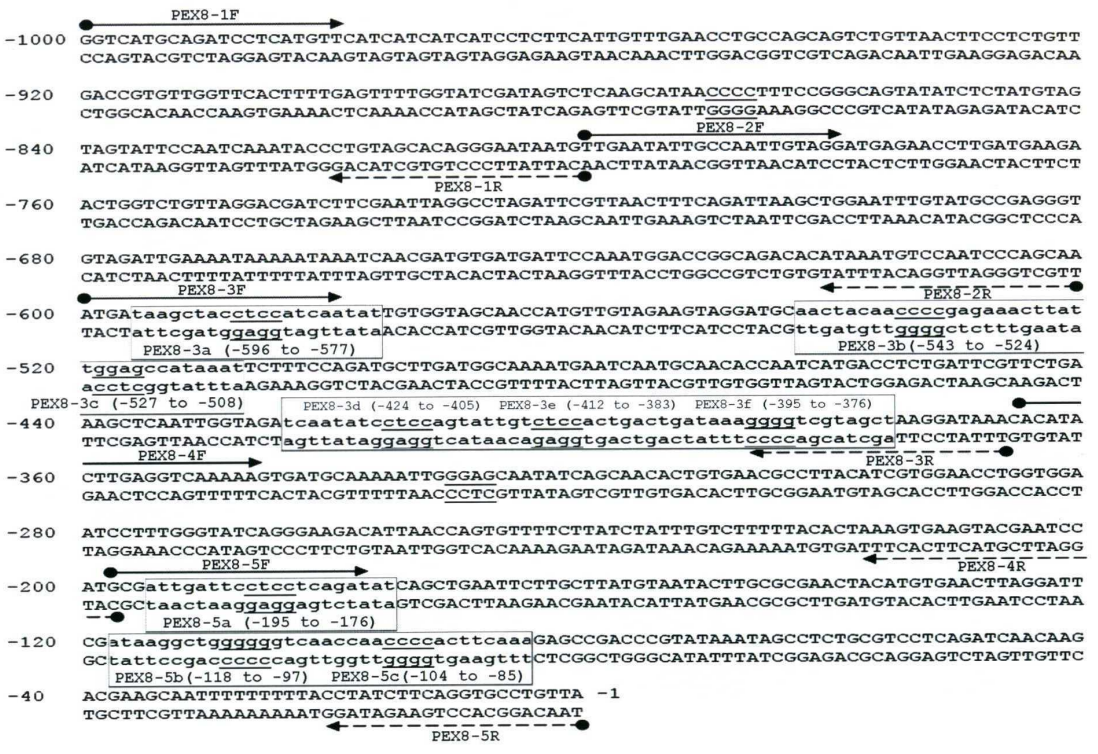


Figure 2. Nucleotide sequence of *PEX8* promoter and strategy for the PCR amplification of promoter DNA fragments. The forward (F) and reverse (R) primers used in PCR reactions to obtain amplicons encompassing five different promoter regions (PEX1-5) are indicated by unbroken and broken arrows, respectively. Oligonucleotides (20 bp) containing the core 5′-CYCC/GGRG-3′ sequence (underlined) used in EMSA studies (Figure 3b) are boxed

the promoter sequences of genes encoding DHAS and PEX8 and identified nucleotides outside the core 5′-CYCC-3′ motif that are essential for Mxr1p binding. Based on these studies, we have derived a consensus sequence for Mxr1p binding.

Materials and methods

Synthesis of oligonucleotides and preparation of radiolabelled DNA probes

Oligonucleotides were purchased from Sigma-Aldrich Chemicals (Bangalore, India). Promoter regions of *DHAS* and *PEX8* were isolated by PCR amplification of *P. pastoris* genomic DNA, using appropriate primers (Figures 1, 2). ^{32}P-labelled DNA probes were generated by radio-labelling the 5′ ends of double-stranded DNA with (γ-^{32}P)ATP and polynucleotide kinase, using standard molecular biology protocols (Sambrook *et al.*, 1989). After radiolabelling, unincorporated radionucleotides were removed by Sephadex G-50 chromatography.

Preparation of recombinant Mxr1p

Overexpression and purification of recombinant Mxr1p has been described recently (Kranthi *et al.*, 2009). This histidine-tagged protein consisting of the first 150 N-terminal amino acids, including the zinc finger DNA-binding domain, is referred to as Mxr1p in this study.

Electrophoretic mobility shift assay (EMSA)

Purified recombinant Mxr1p (10 ng) was incubated with ^{32}P-labelled DNA probes (20 000–40 000 cpm) in a 30 µl reaction containing buffer B (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM

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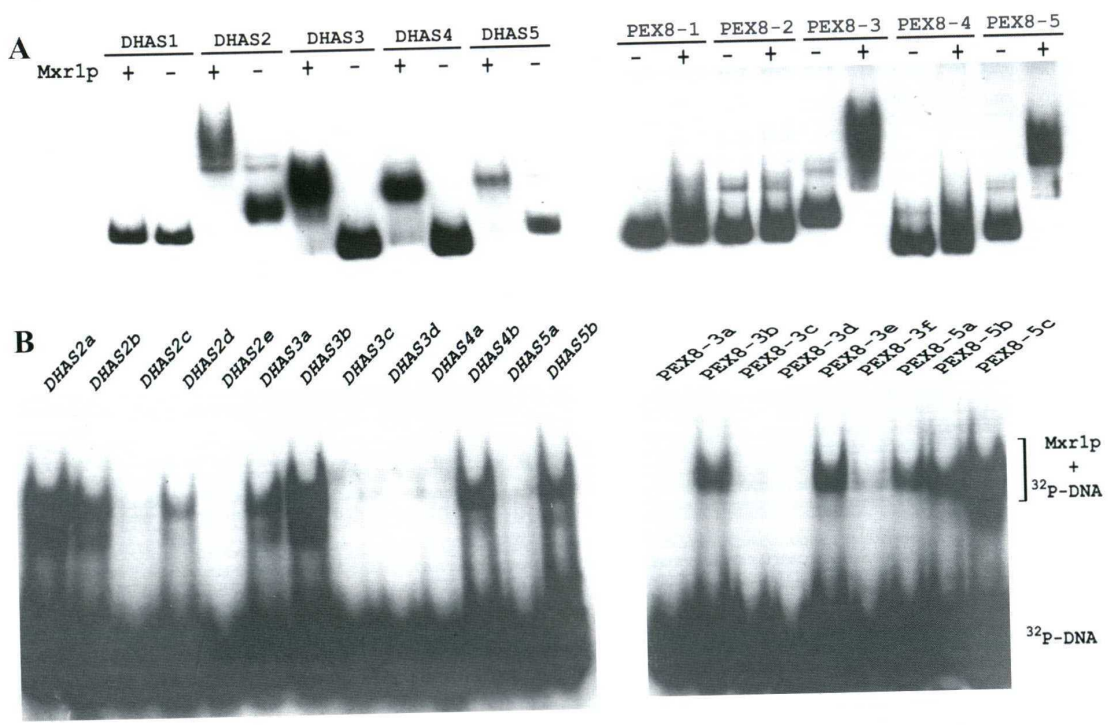


Figure 3. Identification of Mxr1p-binding sites in the promoters of *DHAS* and *PEX8*. (A) Mxr1p-binding to *DHAS* and *PEX8* promoter regions containing varying units of 5′-CYCC-3′ motifs. The promoter DNA fragments used in EMSA are described in Figures 1 and 2. (B) Analysis of Mxr1p-binding to *DHAS* and *PEX8* promoter regions containing a single 5′-CYCC-3′ motif by EMSA. DNA sequences are described in Figures 1 and 2. DNA-binding studies were carried out with ^{32}P-labelled DNA probes (30 000 cpm) and 10 ng Mxr1p

DTT, 100 ng poly(dI–dC), 0.05% NP-40 and 6% glycerol) for 30 min at 4 °C. In some experiments, Mxr1p was incubated first with excess unlabelled oligonuceotides for 5 min prior to the addition of ^{32}P-labelled DNA probes. The reaction mixtures were subjected to electrophoresis at 4 °C on 5% non-denaturing polyacrylamide gel in a buffer containing 7 mM Tris–HCl, pH 7.4, 3 mM boric acid and 1 mM EDTA. The gels were dried and autoradiographed.

Results and discussion

We recently demonstrated that Mxr1p binds at six different regions in the *P. pastoris AOXI* promoter, each of which consists of a core 5′-CYCC-3′ motif (Kranthi *et al.*, 2009). Mutations within this core motif abolishes Mxr1p binding to DNA and this core motif is essential but not sufficient for Mxr1p binding (Kranthi *et al.*, 2009). We reasoned that the core 5′-CYCC-3′ motif may be a common element of Mxr1p-binding sites of promoters of other genes of methanol utilization pathway as well. To examine this, we selected the promoters of genes encoding DHAS and PEX8, since methanol-inducible expression of both these genes is abrogated in *mxr1* mutant strains (Lin-Cereghino *et al.*, 2006). The *DHAS* promoter (−980 to −1 bp) and *PEX8* promoter (−1000 to −1 bp) contain 13 and 11 5′-CYCC-3′ motifs, respectively (Figures 1, 2). To examine whether DNA sequences containing these motifs bind Mxr1p, specific oligonucleotide primers were designed to amplify different promoter regions of *DHAS* and *PEX8* by PCR from *P. pastoris* genomic DNA (Figures 1, 2) to generate PCR products (DHAS1–DHAS5 and PEX8-1–PEX8-5) containing varying units of 5′-CYCC-3′ motifs (Figures 1, 2). These DNA fragments were radiolabelled and their ability to bind to Mxr1p was examined by electrophoretic

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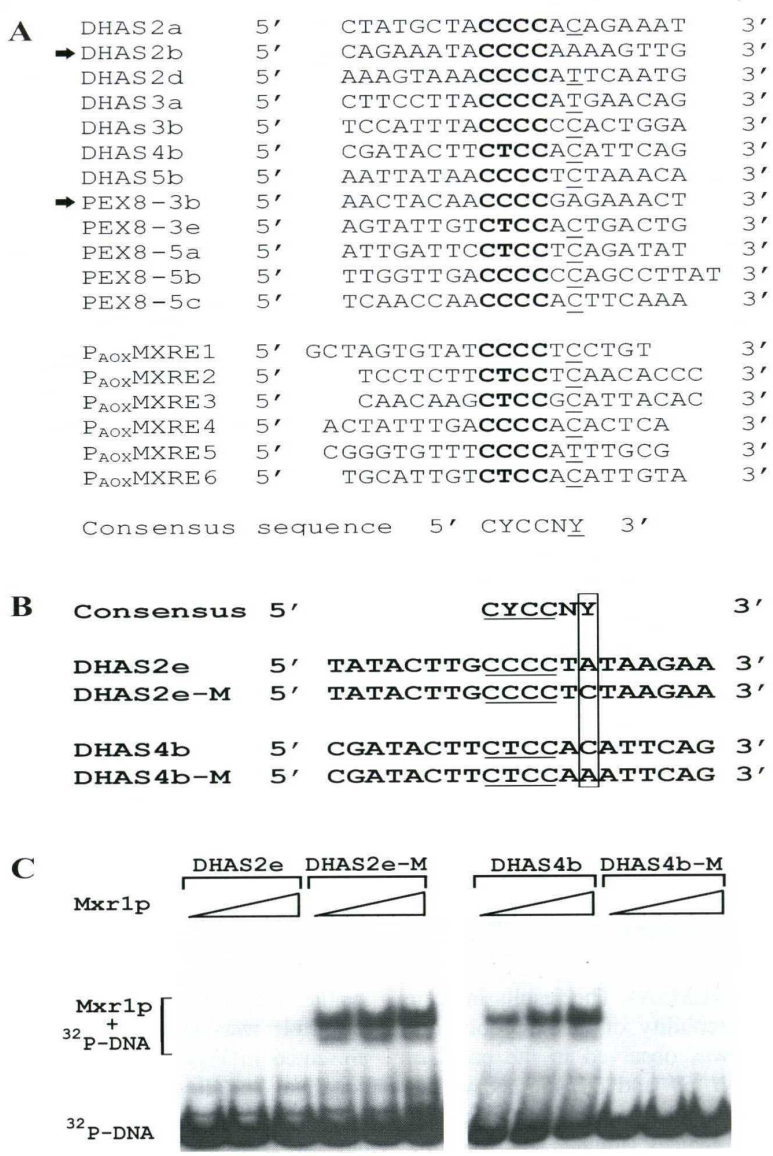


Figure 4. Identification of a consensus sequence for Mxr1p-binding. (A) DNA sequences in the *AOXI*, *DHAS* and *PEX8* promoters that bind to Mxr1p are indicated. The core 5′-CYCC-3′ motif is shown in bold face. The pyrimidine nucleotide which is conserved in all the DNA sequences except DHAS2b and PEX8-3b (arrows) is underlined. The P_{AOX}MXREs have been described recently by Kranthi *et al.* (2009). (B) Nucleotide sequence of DHAS2e, DHAS2e-M, DHAS4b and DHAS4b-M oligonucleotides. The core 5′-CYCC-3′ sequence is underlined. The critical nucleotide outside the core motif vital for Mxr1p-binding is boxed. (C) Demonstration of the importance of the conserved pyrimidine residue outside the core sequence for Mxr1p-binding by EMSA. DNA-binding studies were carried out with ^{32}P-labelled DNA probes (30 000 cpm) and 10 ng Mxr1p. DHAS2e-M represents the DHAS2e sequence carrying the T → A mutation. DHAS2b-M represents the DHAS2e sequence carrying the C → A mutation

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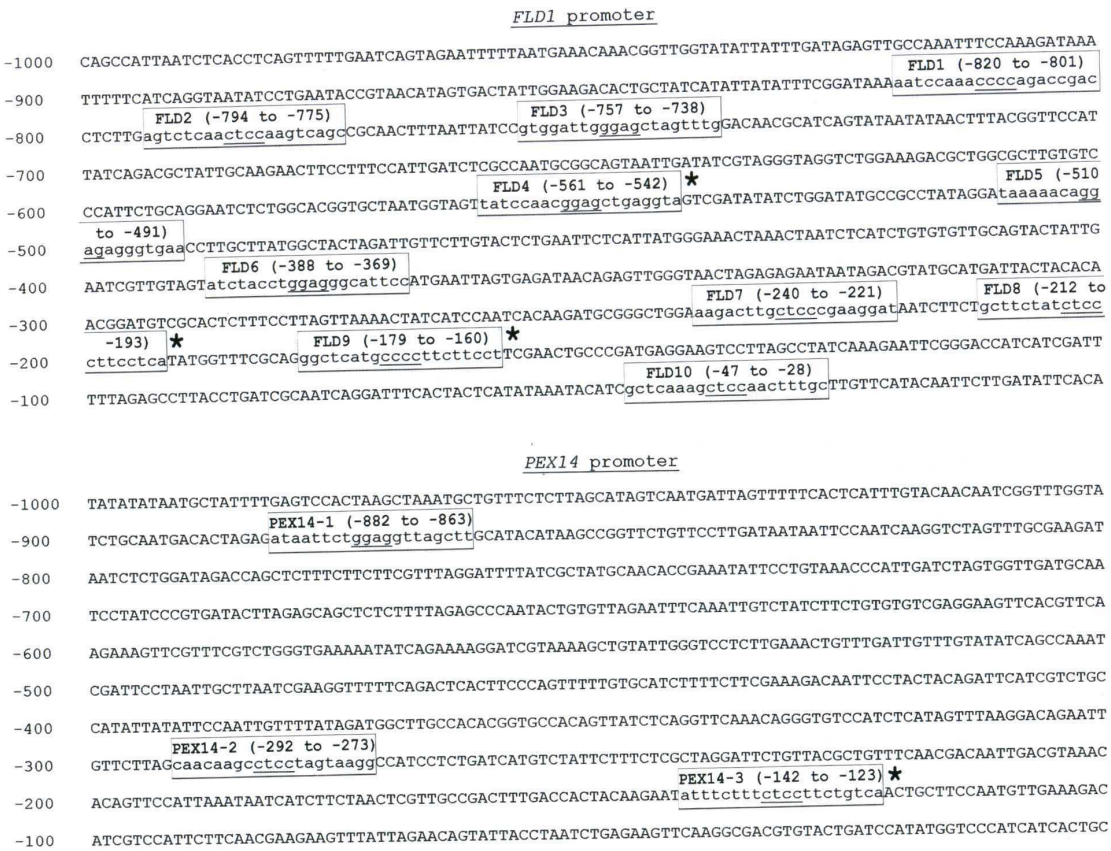


Figure 5. *In silico* analysis of promoters of *FLD1* and *PEX14*. Promoter regions containing the core 5′-CYCC-3′ sequence or its complement (underlined) are boxed. Potential Mxr1p-binding sites which conform to the consensus Mxr1p-binding site (5′-CYCCNY-3′) are marked by *

mobility shift assay (EMSA). The results indicate that a shift in the mobility of DNA probes due to Mxr1p binding was observed in the cases of *DHAS2, DHAS3, DHAS4* and *DHAS5* as well as *PEX8-3* and *PEX8-5* probes, which contain atleast two 5′-CYCC-3′ sequences (Figure 3A). Mobility shift of DNA probes was not observed in case of *DHAS1* and *PEX8-2*, which lack 5′-CYCC-3′ motifs, as well as *PEX8-1* and *PEX8-4*, containing only one 5′-CYCC-3′ motif (Figure 3A). These results are consistent with our earlier studies on the *AOXI* promoter, in which the 5′-CYCC-3′ motif was shown to be an essential component of an Mxr1p-binding site (Kranthi *et al.*, 2009). To further localize Mxr1p-binding sites within these promoter regions, oligonucleotides of ~20 bp length containing 5′-CYCC-3′ sequences were synthesized (Figure 1, 2) and their ability to bind to Mxr1p was evaluated by EMSA. The results presented in Figure 3B indicate that Mxr1p does not bind to all oligonucleotides containing the 5′-CYCC-3′ motif, indicating that, in addition to the 5′-CYCC-3′ motif, additional sequences flanking this core motif also play a key role in Mxr1p binding.

In a recent study based on the analysis of Mxr1p-binding to *AOXI* promoter sequences, we had identified the 5′-CYCC-3′ motif as the consensus sequence for Mxr1p binding (Kranthi *et al.*, 2009). In this study, we examined whether the *DHAS* and *PEX8* promoter sequences which bind Mxr1p conform to this consensus sequence. The results presented in Figure 4A indicate that all the DNA sequences that bind Mxr1p, except

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DHAS2b and PEX8-3b, conform to the sequence 5′-CYCCNC-3′ or 5′-CYCCNT-3′, suggesting that the consensus sequence for Mxr1p binding is 5′-CYCCNY-3′. This was confirmed by modifying the non-binding sequence 5′-CCCCTA-3′ in DHAS2e to 5′-CCCCTC-3′ (DHAS2e-M) (Figure 4B). In an EMSA, Mxr1p-binding was observed only with DHAS2e-M but not DHAS2e (Figure 4C), indicating that DNA sequences containing the 5′-CYCCNY-3′ motif are ideal for Mxr1p binding. Further, Mxr1p binding to DHAS4b is abolished when the sequence 5′-CTCCAC-3′ is modified to 5′-CTCCAA-3′. Thus, the inability of Mxr1p to bind to PEX8-4 can now be explained based on the fact that it contains the 5′-CTCCCA-3′ rather than the 5′-CTCCCY-3′ motif. However, DHAS2c, DHAS3d and PEX8-3a as well as PEX8-1 are exceptions, as they do not bind Mxr1p despite containing the 5′-CYCCNY-3′ motif, suggesting that DNA elements other than those identified in this study may also play a role in Mxr1p-binding to DNA. The strategy for identification of Mxr1p-binding sites described in this study is simple and versatile and can be used for the identification of Mxr1p-binding sites in any promoter of interest. Based on this study, we have carried out an *in silico* analysis of promoters of *FLD1* and *PEX14* genes and identified potential Mxr1p-binding sites within 1.0 kb upstream region (Figure 5). Promoter constructs carrying specific mutations in each of these Mxr1p-binding sites can be examined for their ability to activate reporter gene expression, and such studies can pave way for the identification of Mxr1p-binding sites that function as methanol response elements *in vivo*.

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