Chapter 3
Hansenula polymorpha (Pichia angusta): Biology and Applications

Gotthard Kunze, Hyun Ah Kang, and Gerd Gellissen

Contents

Abstract *Hansenula polymorpha* (*Pichia angusta*) belongs to a limited number of methylotrophic yeast species. It is able to assimilate nitrate and can grow on a range of carbon sources. Furthermore, *H. polymorpha* is a thermo-tolerant microorganism with some strains growing at temperatures up to 50°C and more. These unusual characteristics render *H. polymorpha* attractive as a model organism to study the development and functions of peroxisomes and the biochemistry of nitrate assimilation. *H. polymorpha* provides an established platform for heterologous gene expression and is distinguished by an impressive track record as producer of recombinant proteins that include commercially available pharmaceuticals like hepatitis B vaccine, insulin and the IFNα-2a.

Keywords Methylotrophic yeast, nitrate, thermotolerant, peroxisomes, recombinant proteins

T. Satyanarayana, G. Kunze (eds.), *Yeast Biotechnology: Diversity and Applications,*
DOI: 10.1007/978-1-4020-8292-4_3, © Springer Science+Business Media B.V. 2009

3.1 History, Phylogenetic Position, Basic Genetics and Biochemistry of *H. polymorpha*

A limited number of yeast species are able to utilize methanol as a sole energy and carbon source. The range of methylotrophic yeasts includes *Candida boidinii, Pichia methanolica, Pichia pastoris* and *Hansenula polymorpha*, all of them provide attractive platforms for heterologous gene expression (Gellissen, 2000). Especially *H. polymorpha* has found successful application in industrial production of heterologous proteins as detailed later (Gellissen, 2002; Guengerich et al., 2004). In basic research, it is used as a model organism for peroxisomal function and biogenesis as well as nitrate assimilation (Gellissen and Veenhuis, 2001; van der Klei and Veenhuis, 2002; Siverio, 2002; Kang et al., 2002). The presence of a nitrate assimilation pathway is a feature not shared by the other methylotrophs. Since *H. polymorpha* is the most thermo-tolerant representative of this group, it might also be better suited as source and for the production of proteins considered for crystallographic studies. The first methylotrophic yeast described was *Kloeckera* sp. No 2201, later re-identified as *Candida boidinii* (Ogata et al., 1969). Subsequently the other species, including *H. polymorpha*, have been identified as methanol-assimilating yeasts (Hazeu et al., 1972).

Three basic *H. polymorpha* strains with unclear relationships and of independent origins have been used in basic research and in the production of recombinant proteins, which exhibit different features. The strain CBS4732 (CCY38-22-2; ATCC34438, NRRL-Y-5445) was initially isolated from irrigated soil near a distillery in Pernambuco, Brazil (Morais and Maia, 1959); DL-1 (NRRL-Y-7560; ATCC26012) was also isolated from soil by Levine and Cooney (1973); and NCYC495 (CBS1976; ATAA14754, NRLL-Y-1798) was obtained from spoiled concentrated orange juice in Florida, which was initially designated as *Hansenula angusta* (Wickerham, 1951). The strains CBS4732 and NCYY495 can be mated, while the strain DL-1 cannot be mated with the other two (K. Lahtchev, personal communication).

The genus *Hansenula* H. et P. Sydow includes ascosporogenic yeast species exhibiting spherical, spheroidal, ellipsoidal, oblong, cylindrical, or elongated cells. One to four ascospores are formed. Ascigenic cells are diploid arising from conjugation of haploid cells. The genus is predominantly heterothallic. *H. polymorpha* is probably homothallic exhibiting an easy inter-conversion between the haploid and diploid state (Teunisson et al., 1960; Middelhoven, 2002). After performing DNA/DNA reassociation studies, it was proposed to merge both genera and transfer *Hansenula* species with hat-shaped ascospores to *Pichia* Hansen emend Kurtzman (Kurtzman, 1984), although *Hansenula* spp. can grow on nitrate and *Pichia* spp. cannot. Kurtzman and Robnett (1998) provided a phylogenetic tree in which nitrate-positive and nitrate-negative *Pichia* are clustered, demonstrating the unreliability of nitrate assimilation for prediction of kinship. The leading taxonomy monographs follow this proposal, re-naming *H. polymorpha* as *Pichia angusta* (Kurtzman and Fell, 1998; Barnett et al. 2000). However, the merging of the genera

is still criticized by some taxonomists, and there are arguments for maintaining the established and popular name *Hansenula polymorpha* (Middelhoven, 2002; Sudbery, 2003) (Fig. 3.1).

Some strains of *H. polymorpha* can tolerate temperatures of 49°C and higher (Teunisson et al., 1960; Reinders et al., 1999). Cells grown under conditions of elevated temperature accumulate trehalose as thermo-protective compound. It was shown that trehalose synthesis is not required for growth under these conditions, but for acquisition of thermo-tolerance (Reinders et al., 1999). The synthetic steps for trehalose synthesis have been detailed for *H. polymorpha* and *TPS1,* the key enzyme gene of this pathway, has been isolated and characterized (Romano, 1998; Reinders et al., 1999). Transcripts of this gene encoding trehalose-6-phosphate synthase were found to be very abundant in cells grown at elevated temperature, but to be present in high quantities when grown even at normal temperature (Reinders et al., 1999). The *TPS1*-derived promoter provides an attractive element to drive constitutive heterologous gene expression which can be further boosted at temperatures above 42°C (Amuel et al., 2000; Suckow and Gellissen, 2002).

All methylotrophic yeasts share an identical methanol utilization pathway (Tani, 1984; Yurimoto et al., 2002; Kang and Gellissen, 2005) (Fig. 3.2).

Growth on methanol is accompanied by a massive proliferation of peroxisomes in which the initial enzymatic steps of this pathway take place (Gellissen and Veenhuis, 2001; van der Klei and Veenhuis, 2002; Yurimoto et al., 2002). During growth on methanol key enzymes of the methanol metabolism are present in high amounts. An especially high abundance can be observed for MOX, FMD, and DHAS (Gellissen et al., 1992a). Their presence is regulated at the transcriptional level of the respective genes. In the related species, *C. boidinii*, *P. methanolica*, and *P. pastoris*, this gene expression strictly depends on the presence of methanol or methanol derivatives (Gellissen, 2000), whereas in *H. polymorpha* strong expression is elicited by appropriate levels of glycerol or under conditions of glucose starvation (Kang and Gellissen, 2005). This expression profile has been confirmed by micro-array analysis of transcripts isolated from cells grown under the respective

Eukaryota **(Superkingdom)**
Fungi **(Kingdom)**
Ascomycota **(Phylum)**
Saccharomycotina **(Subphylum)**
Saccharomycetes **(Class)**
Saccharomycetales **(Order)**
Saccharomycetaceae **(Family)**
Pichia **(Genus)**
Pichia angusta **(Species)**

Fig. 3.1 Taxonomy of *P. angusta* (synonym: *H. polymorpha*) (Kurtzman and Fell, 1998; Barnett et al. 2000)

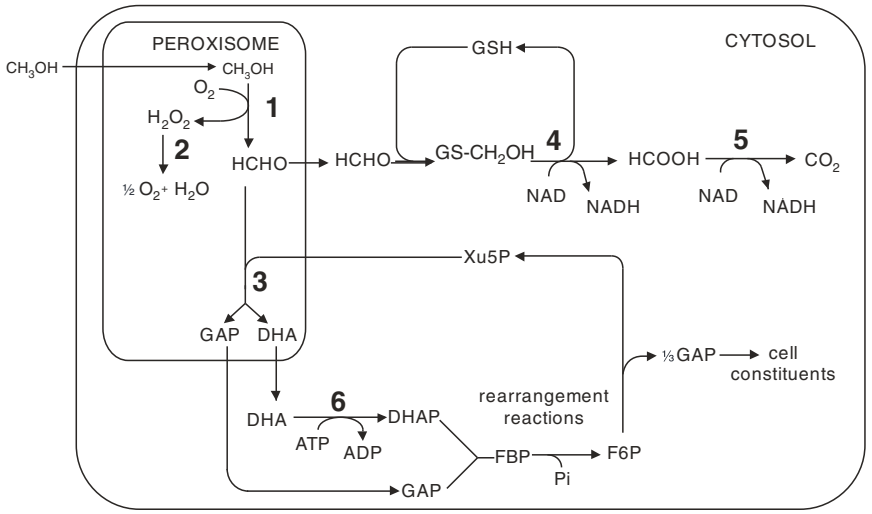

Fig. 3.2 The methanol utilization pathway and its compartmentalization in methylotrophic yeasts (modified after Kang and Gellissen 2005). (1) Methanol is oxidized by alcohol oxidase to generate formaldehyde and hydrogen peroxide. (2) The toxic hydrogen peroxide is decomposed by catalase to water and oxygen. (3,4) Within a dissimilatory pathway the formaldehyde is oxidized by two subsequent dehydrogenase reactions to carbon dioxide, catalyzed by a formaldehyde dehydrogenase (FLD) and a formate dehydrogenase (FMD or FDH). (5) For assimilation the formaldehyde reacts with xylulose-5-phosphate (Xu_5P) by the action of dihydroxyacetone synthase (DHAS) to generate the C_3 compounds glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone (DHA). (6) DHA is phosphorylated by dihydroxyacetone kinase (DHAK) to dihydroxyacetone phosphate (DHAP). (7) GAP and DHAP yield in an aldolase reaction fructose 1,6-biphosphate (FBP). (8) In further steps of the pentose phosphate cycle fructose-5-phosphate and xylulose-5-phosphate are finally generated

conditions (H.A. Kang, personal communication). Expression of methanol utilization pathway genes is thus subject to de-repression mechanism in absence of glucose in *H. polymorpha*, but to a methanol-dependent induction mechanism in all other methylotrophs conferred by inherent properties of the respective promoters. Again these promoter elements constitute strong components for the control of heterologous gene expression that can be regulated by carbon source addition. In *H. polymorpha* the elements derived from the *MOX* and the *FMD* genes are particularly attractive in providing the unique possibility of eliciting high promoter activity without methanol addition to a culture medium.

Identified and characterized genes of the *H. polymorpha* methanol utilization pathway are boxed and are shown in the pathway position of the encoded enzymes. The genes are *MOX* (Ledeboer et al. 1985), *DAS* (Janowicz et al., 1985), *CAT* (Didion and Roggenkamp 1992), *DAK* (Tikhomirova et al., 1988), *FLD1* (Baerends et al., 2002), and *FMD* (Hollenberg and Janowicz, 1988).

3.2 Strains

From the three *H. polymorpha* parental strains, NCYC495, CBS4732, and DL-1 (see previous section), some 50 other strains have been derived. DL-1 strains are not employed in classical genetic analyses. The inability of the DL-1 strain to copulate makes this strain inconvenient for classical genetic manipulation exploiting meiotic segregation. However, the relatively high frequency of homologous recombination in the DL-1 strain enables application of several molecular genetic techniques developed in *S. cerevisiae* to the DL-1 strain. The DL-1 strain has certain advantages in that it has a higher growth rate and adapts more quickly to culture media than the other parental strains; additionally DL-1 strains have a higher frequency of homologous recombination than other strains (Kang et al., 2002; Lahtchev, 2002). Several host strains suitable for heterologous protein expression, including auxotrophic mutants, protease-deficient strains, and *mox*-negative strains, have been constructed in the DL-1 strain mostly using gene disruption techniques. A pop-out cassette using *HpURA3* as a selection marker has been constructed to recover the auxotrophic marker for the subsequent gene disruption or for subsequent transformation with expression vectors (Kang et al., 2002). A similar range of auxotrophic mutants are derived from the CBS4732 background, mostly using chemical mutagenesis methods and mating techniques (Lahtchev, 2002).

Most classical genetic techniques have been performed using NCYC495, which shows mating and sporulation (Lahtchev, 2002). Unlike the other two parental strains, NCYC495 does not grow well on methanol-containing media and therefore does not have the strong methanol pathway-derived promoters available to the other strains for gene expression. Instead, NCYC495 has other interesting applications, including its employment for the study of nitrate assimilation mentioned before (Siverio, 2002). Cells from CBS4732 grow well on methanol and show strong mating and sporulation (Lahtchev, 2002). Both CBS4732 and DL-1 and their derivatives are employed in the production of recombinant products (Kang et al., 2001a; Gellissen, 2000, 2002; Müller et al., 2002; Park et al., 2004, see also the forthcoming section of this chapter). In contrast to DL-1 strains, some sub-strains of CBS4732

Table 3.1 Comparison of selected gene sequences from *H. polymorpha* strains CBS4732 and DL-1

Gene name	Amino acid identity (%)	Nucleic acid identity (%)	Accession no.*	Reference
CST13	96.7%	95.8%	AF454544	Kim et al., 2002
CPY	98.0%	95.9%	U67174	KRIBB
GSH2	96.0%	94.5%	AF435121	KRIBB
MNN9	96.3%	95.5%	AF264786	Kim et al., 2001
PMI40	97.9%	94.9%	AF454544	Kim et al., 2002
PMR1	98.5%	95.2%	U92083	Kang et al., 1998a, b
YPT1	99.5%	97.2%	AF454544	Kim et al., 2002
Average	97.6%	96.6%		

*The sequences of genes isolated from the DL-1 strain were obtained from GenBank and compared with those from the CBS4732 strain (Ramezani-Rad et al., 2003).

are not easily applied to recombinatory methods, perhaps due to their high mitotic stability (Suckow and Gellissen, 2002). For a selection of *H. polymorpha* strains and for protocols specific to parental strains, see Degelmann et al. (2002) (Table 3.1).

3.3 *N*-Linked Glycosylation in *H. polymorpha*

Initial processing of *N*-linked glycans on glycoproteins occurs in the endoplasmic reticulum (ER) of eukaryotes and results in the core oligosaccharide, $Man_8GlcNAc_2$. Further maturation of oligosaccharides in the Golgi apparatus is quite variable among yeast species (Gemmill and Trimble, 1999). Yeasts elongate the core oligosaccharide mostly by addition of mannose, leading to the formation of core-sized structures ($Man <_{15} GlcNAc_2$) as well as hypermannose structures ($Man_{50-200}GlcNAc_2$) with extended poly-α-1,6 outer mannose chains, which are decorated with various carbohydrate side chains in a species-specific manner. In *S. cerevisiae*, the linear backbone of the outer chain is often composed of 50 or more mannoses, highly branched by addition of α-1,2-linked mannoses and terminally capped with α-1,3-linked mannoses, generating heavily hypermannosylated glycoproteins. α-1,3-linked terminal mannose are considered to cause allergenic reactions. The outer chain also contains several mannosylphosphate residues, providing the oligosaccharide with a negative net charge (Jigami and Odani, 1999; Kim et al., 2004). Compared to *S. cerevisiae*, the mannose outer chains of *N*-linked oligosaccharides generally appear to be much shorter in *H. polymorpha* (Kang et al., 1998b), although extensive hyperglycosylation has also been reported in a few cases of recombinant glycoproteins produced in this yeast (Müller et al., 1998). A recent study on the structure of the oligosaccharides derived from the recombinant *Aspergillus niger* glucose oxidase (GOD) and the cell wall mannoproteins derived from *H. polymorpha* has revealed that most oligosaccharide species attached to the recombinant GOD have core-sized structures ($Man_{8-12}GlcNAc_2$) without terminal α-1,3-linked mannose residues (Kim et al., 2004). Therefore, the outer chain processing in the *N*-linked glycosylation pathway in *H. polymorpha* appears to be similar to that in *P. pastoris*, with the addition of shorter outer chains to the core and no terminal α-1,3-linked mannose addition (Montesino et al., 1998; Bretthauer and Castellano, 1999).

Differences between yeast and mammalian N-glycosylation are a major limitation for yeasts to be used in the production of recombinant glycoproteins for therapeutic use. Glycoproteins derived from yeast expression systems contain *N*-glycans of the high mannose type, in humans both *N*-glycans of a high mannose type and of a terminally sialylated complex type exist. Attempts have been made to genetically modify glycosylation processes in *S. cerevisiae* (Chiba et al., 1998) and *P. pastoris* (Callewaert et al., 2001) to trim the yeast *N*-glycans of the high mannose type to the human glycans of the $(Man)_5$-$(GlcNAc_2)$ intermediate type. A more advanced achievement has been made to genetically re-engineer the glycosylation pathway of *P. pastoris* to produce the complex human *N*-glycan *N*-acetylglucosamine$_2$-mannose$_3$-*N*-acetylglucosamine ($GlcNAc_2Man_3GlcNAc_2$) (Hamilton

et al., 2003). Recently, EPO could be produced in an authentic form with the addition of sialylated complex *N*-glycans (Hamilton et al., 2006). In *H. polymorpha* genes of N-glycosylation have been characterized and successful approaches have been taken to replace such genes by those from other fungal organisms to re-engineer strains adding humanized N-glycans of the intermediate mannose type (Kim et al., 2006). Co-expression of secretory pathway genes has resulted in a reduced extent of over-glycosylation (co-expression of a *S. cerevisiae*-derived *CMK2* gene) in case of a secreted recombinant phytase or in the presence of N-glycan structures corresponding in size to core-glycosylation (co-expression of a *H. polymorpha* –derived *CNE1* gene) in case of secreted IFNγ (Steinborn et al., 2006).

3.4 Characteristics of the *H. polymorpha* Genome

The entire genome of strain CBS4732 (CCY38-22-2; ATCC34438; NRRL-Y-5445) has been characterized (Ramezani-Rad et al., 2003), for the other biotechnologically applied strain DL-1 (NRRL-Y-7560; ATCC26012) data on karyotyping are available (Kang and Gellissen, 2005). Pulsed-field gel electrophoresis of *H. polymorpha* chromosomes revealed that both strains have 6 chromosomes, ranging from 0.9 to 1.9 Mbp, but the electrophoretic patterns of their chromosomes are quite different. The sequence identity of the open reading frame for the selected genes ranges between 94.5–97.2%, with an average value of 96.6%. The sequence differences are observed to be much more magnificent at the 5′- and 3′-untranslated regions, which might be involved in controlling gene expression. This implies that the two strains are closely related but have distinct genetic and physiological characteristics (Ramezani-Rad et al., 2003).

For sequencing of *H. polymorpha* of strain CBS4732, a BAC library with approximately 17x coverage was constructed in a pBACe3.6 vector (Osoegawa et al., 1998, 1999). Sequencing resulted in the characterization of 8.733 million base pairs assembled into 48 contigs. The derived sequence covers over 90% of the estimated total genome content of 9.5 Mbp located on 6 chromosomes which range in size between 0.9 and 2.2 Mbp. Out of the sequenced 8.73 Mb 5848 ORFs have been extracted for proteins longer than 80 amino acids. 389 ORFs smaller than 100 amino acids were identified. 4771 ORFs have homologues to known proteins (81.6%). Calculation of the gene density and protein length, taking into account the gene numbers, showed an average gene density of 1 gene/1.5 kb and an average protein length of 440 amino acids. 91 introns have been identified by homology to known proteins and confirmed by using GeneWise (Birney and Durbin, 2000). 80 tRNAs were identified, corresponding to all 20 amino acids. From approximately 50 rRNA clusters 7 clusters have been fully sequenced. All clusters are completely identical and have a precise length of 5033 bp (Table 3.2).

The main functional categories and their distribution in the gene set are manually predicted for energy, 4%; cellular communication, signal transduction mechanism, 3%; protein synthesis, 6%; cell rescue, defense and virulence, 4%; cellular

Table 3.2 *H. polymorpha* CBS4732 genome statistics

Contigs: 48	48
Total length of contigs:	8,733,442 bp
Average contig length:	182 kb
No. of extracted ORFs:	5,848
No. of ORFs < 100 aa:	389
Average gene density:	1 gene/1.5 kb
Average gene size (start-stop):	1.3 kb (1,320 nt)
Average protein length:	440 aa

Table 3.3 Functional categorization of genes

Functional category	No. ORFs	%
Metabolism	1114	19
Energy	231	4
Cell growth, Cell division and DNA synthesis	518	9
Transcription	767	13
Protein synthesis	323	6
Protein destination	1014	17
Transport facilitation	423	7
Cellular transport and transport mechanisms	518	9
Control of cellular organization	417	7
Cellular communication/signal transduction	170	3
Cell rescue, defense, and virulence	260	4
Cell fate	282	5
Regulation of/interaction with cellular environment	184	3

transport and transport mechanisms, 9%; cell cycle and DNA processing, 9%; protein fate (folding, modification, destination) 17%; transcription, 13% and metabolism, 19% (Ramezani-Rad et al., 2003) (Table 3.3).

Based on the sequence data a genome micro-array has been established (Oh et al., 2004; Park et al., 2007) that is currently applied to transcript profiling of *H. polymorpha*, among others to carbon source-dependent gene expression as pointed out before.

3.5 The *H. polymorpha*-Based Expression Platform

Generation of recombinant *H. polymorpha* strains of a CBS4732 background typically employs vectors that are mitotically stable integrated into the genome of the host (Gellissen and Hollenberg, 1997). The traditional vectors applied to transformation are provided as circular plasmids. Despite the presence of HARS or S. *cerevisiae*–derived ARS sequences they are integrated and not of an episomal fate. These plasmids may integrate into the host DNA over a number of generations, resulting in strains with as many as 100 integrated plasmids present in tandem repeats (Gellissen, 2000; Kang et al., 2002). Obviously the circular plasmids are not randomly integrated

as initially postulated but recombine with genomic sequences represented on the vector. This was shown with a particular vector harboring a *FMD* promoter/*HBsAg* fusion where recombination within the *FMD* gene was observed (Dahlems U, personal communication). It seems most likely that homologous recombination also takes place with vectors equipped with *MOX*, *TPS1* and other promoter elements. A high number of integrated copies are not always a prerequisite for high-level expression, especially in the case of secretory protein production. In a particular case, four copies of a HARS vector were sufficient to obtain efficient production of a *Schwanniomyces occidentalis*-derived glucoamylase in *H. polymorpha* CBS (Gellissen et al., 1992b). Other examples are *H. polymorpha* DL-1-based production strains for human urinary-type plasminogen activator (u-PA) and human serum albumin (HSA). In these cases, a single- or two-copy integration of the expression vector resulted in the maximal levels of recombinant u-PA or HSA secreted into culture supernatants (Kang et al., 2002). Targeted integration into sites others than the highly expressed methanol pathway genes require much longer target gene overlaps than those used in *S. cerevisiae* (Gonzalez et al., 1999; van Dijk et al., 2001). Plasmids harboring one of a set of several cloned sub-telomeric ARS sequences derived from the DL-1 strain have been described. These vectors homologously integrate into a genomic counterpart and result in recombinant strains harbouring single or multiple tandemly repeated copies at the respective sub-telomeric genomic locus (Sohn et al., 1999; Kim et al., 2003). A set of vectors has been constructed to target the heterologous DNA to the rDNA locus of *H. polymorpha*. *Arxula adeninivorans* and *H. polymorpha*-derived rDNA targeting elements have been defined for optimal integration and expression characteristics when present on wide-range expression/integration vectors. They are included in the wide-range yeast (CoMed™) system described elsewhere in the book (Steinborn et al., 2005, 2006).

After transformation, cells are plated on selective media according to the requirements of the selection marker gene contained on the plasmid. Macroscopic colonies typically appear after 4–5 days of incubation at 37°C. Colonies are then transferred within a ‘passaging step’ to liquid selective medium and incubated under vigorous shaking for 24 h to 48 h at 37°C. An aliquot of the dense culture is then used to inoculate fresh selective medium, and the incubation is repeated. After 3–8 subsequently applied passaging steps, the initially episomal plasmid is integrated into the genome.

For heterologous gene expression in *H. polymorpha* a range of homologous and heterologous promoters of extraordinary strength is available. *MOX* and *FMD* promoters are derived from genes of the methanol degradation pathway that can be carbon source-regulated as described before. The *TPS1* promoter, derived from the trehalose 6-phosphate synthase gene of *H. polymorpha*, is constitutive with regard to different carbon sources and can be influenced by different temperatures (Amuel et al., 2000). In combination with a high copy numbers of the integrated plasmid these strong promoters can provide very high expression rates of the heterologous gene in selected strains. For secreted phytase, product levels of up to 13.5 g l^{-1} have been obtained. This extremely high productivity was elicited by the *FMD* promoter applying conditions of glucose starvation to culturing (Mayer et al., 1999). In addition

to homologous promoters elements derived from alternative yeasts can be employed. A particularly attractive component is the strong constitutive *A. adeninivorans*-derived *TEF1* promoter (Steinborn et al., 2006). Other available but less frequently applied regulative promoters are derived from inducible genes encoding enzymes involved in nitrate assimilation (e.g. *YNT1*, *YNI1*, *YNR1*, which can be induced by nitrate and repressed by ammonium - Avila et al., 1998), or the enzyme acid phosphatase (the *PHO1* promoter - Phongdara et al., 1998), and the *FLD1* promoter, yet another element derived from a methanol utilization pathway gene (Baerends et al., 2002). Examples of constitutive promoters are *ACT* (Kang et al., 2001b), *GAP* (Heo et al., 2003), and *PMA1* (Cox et al., 2000). The *PMA1* promoter even competes with the outstanding *MOX* promoter in terms of high expression levels; *PMA1* provides an attractive element for the co-expression of genes on industrial scale (Cox et al., 2000).

Signal sequences may be fused to the target open reading frame (ORF) for direct release of synthesized proteins directly into the media, or into a pre-selected cell compartment, such as the peroxisome, the vacuole, or for targeting to the cell surface. For ER-mediated secretion a range of pre-leader sequences such as that derived from the *PHO1* gene encoding a repressible acid phosphatase (Phongdara et al., 1998) and a *S. occidentalis*-derived *GAM1* sequence (van Djik et al., 2000; Weydemann et al., 1995) are available. Most commonly, the *S. cerevisiae*-derived the MFα1 pre-pro- leader is employed as the preferred component (Brake et al., 1984; Gellissen, 2000) Available targeting signals include PTS1 and PTS2 for addressing the peroxisome (van Djik et al., 2000); glycosylphosphatidylinositol (GPI)-anchoring motifs derived from the GPI-anchored cell surface proteins, such as *HpSED1*, *HpGAS1*, *HpTIP1*, and *HpCWP1*, have been exploited to develop a cell surface display system in *H. polymorpha*. When the recombinant glucose oxidase (GOD) was produced as a fusion protein to these anchoring motifs, most enzyme activity was detected on the cell surface (Kim et al., 2002).

Expression and integration vectors in *H. polymorpha* are composed of prokaryotic and yeast DNA incorporating elements described before or combinations thereof (Gellissen and Hollenberg, 1997). Vectors are either supplied as circular plasmid or linearized and targeted to a specific genomic locus. Possible targets for homologous integration include the *MOX*/*TRP* locus (Agaphonov et al., 1995), an ARS sequence (Agaphonov et al., 1999; Sohn et al., 1996), the *URA3* gene (Brito et al., 1999), the *LEU2* gene (Agaphonov et al., 1999), the *GAP* promoter region (Heo et al., 2003), or the rDNA cluster (Klabunde et al., 2002, 2003). In the novel wide-range yeast vector (CoMed™) vector system individual modules consisting of expression cassettes equipped with attractive promoters of choice, selection markers, rDNA targeting sequences or ARS sequences can be combined (Steinborn et al., 2006) (Fig. 3.3).

For detailed information of various traditional vectors the reader is referred to previous publications (Suckow and Gellissen, 2002; Kang et al., 2002; Guengerich et al., 2004). Plasmids that have been successfully developed for industrial use of CBS4732-based strains include pFPMT121 for *FMD* promoter-controlled production of phytase (Mayer et al., 1999; Suckow and Gellissen, 2002) and a derivative of pMPT121 for *MOX* promoter-controlled production of the anti-coagulant hirudin (Avgerinos et al., 2001; Suckow and Gellissen, 2002).

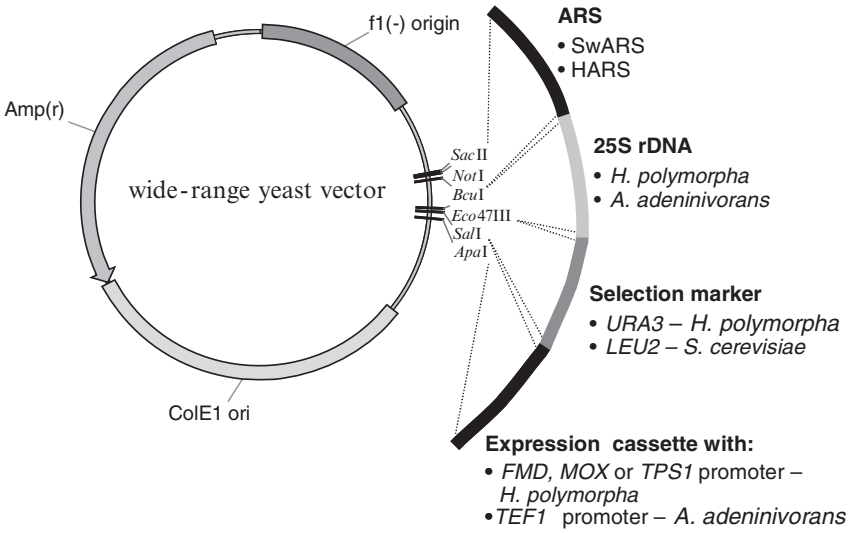

Fig. 3.3 General design of the wide-range yeast vector (CoMed™) with elements to address *H. polymorpha.* The vector contains all *E. coli* elements for propagation in the *E. coli* system and a MCS for integration of ARS, rDNA, selection marker and expression cassette modules. For this purpose, ARS fragments are flanked by *Sac*II and *Bcu*I restriction sites, rDNA regions by *Bcu*I and *Eco*47III restriction sites, selection markers by *Eco*47III and *Sal*I restriction sites and promoter elements by *Sal*I and *Apa*I restriction sites

3.6 Product and Process Examples

We now briefly summarize a few industrially relevant examples of *H. polymorpha*-based processes, all of them leaning on CBS4732-derived strains employing *MOX* or *FMD* promoters for expression control of the foreign gene. More detailed description of fermentation and purification procedures can be found in recent articles (Jenzelewski, 2002; Hellwig et al., 2005). The design of a fermentation procedure greatly depends on characteristics of the host cell, the intended routing of the recombinant gene product, and most importantly on the promoter elements used. The commonly used culture media are based on simple synthetic components. They contain trace metal ions and adequate nitrogen sources, which are required for efficient gene expression and cell yield, but no proteins. The total fermentation time varies between 60 and 150 hours. Due to the inherent versatile characteristics of the two methanol pathway-derived promoters fermentation modes vary, for the most part in the supplemented carbon source: glycerol, methanol, glucose, and combinations thereof may be selected.

In processes for secretory heterologous proteins usually a ‘one-carbon source’ mode is employed supplementing the culture medium with glycerol only. A hirudin production process may serve as an example for this fermentation mode. In this process a strain was employed that harbours 40 copies of an expression cassette for an *MFα1* prepro-sequence/ hirudin fusion gene under control of the *MOX* promoter (Weydemann et al., 1995; Avgerinos et al., 2001;

Bartelsen et al., 2002). Hirudin production was promoted by reducing initial glycerol concentration and maintaining it on a suitable level by a pO_2-controlled addition of the carbon source. The fermentation is started with 3% (w/v) glycerol at the beginning of fermentation. After consumption of the carbon source after 25 hours the pO_2-controlled feeding mode is initiated resulting in a glycerol concentration between 0.05 and 0.3% (w/v) (derepression of the *MOX* promoter). The fermentation run is terminated after 36 hours of derepression (total fermentation time of 72 hours). Then the broth is harvested and the secreted product is purified from the supernatant by a sequence of ultrafiltration, ion exchange, and gel filtration steps.

In case of HBsAg production, a 'two carbon source' fermentation mode was employed (Brocke et al., 2005). The producer strain harbours high copy numbers of an expression cassette with the coding sequence for the small surface antigen (S-antigen) under control of methanol pathway promoters. The selected strain is fermented on a 50 l scale. The product-containing cells are generated via a two fermenter cascade, consisting of a 5 l seed inoculating the 50 l main fermenter. The initial steps of fermentation closely follow those described for the production of hirudin. Cultivation is started with a glycerol feed in a fed-batch mode, to be followed by subsequent semi-continuous glycerol feeding controlled by the dissolved oxygen level in the culture broth. This de-repression phase is then followed by a batchwise feeding with methanol in the final fermentation mode. The product concentration increases to amounts in the multigram range. It consists of a lipoprotein particle in which the recombinant HBsAg is inserted into host-derived membranes. Addition of methanol also serves for the proliferation of organelles and consequently for the synthesis and proliferation of membranes. Methanol is thus needed in this case to provide a high-yield and balanced co-production of both components of the particle. For downstream processing the harvested cells are disrupted and the particles are purified in a multi-step procedure that includes adsorption of a debris-free extract to a matrix and the subsequent application of a sequence of ion exchange, ultra-filtration, gel filtration, and ultra-centrifugation steps (Schaefer et al., 2001, 2002; Brocke et al., 2005).

For the production of phytase *H. polymorpha* has been used in a particularly efficient process (Mayer et al., 1999; Papendieck et al., 2002), a prerequisite for an economically competitive production of a technical enzyme. A strain was generated in which the phytase sequence is under control of the *FMD* promoter. A fermentation procedure was developed to achieve high levels of enzyme production. The active status of the *FMD*-promoter was maintained by glucose starvation (fermentation with minimal levels of continuously fed glucose). Strains were found to produce the recombinant phytase at levels ranging up to 13.5 g l^{-1} (Mayer et al., 1999). The secreted product is purified through a series of steps, including flocculation centrifugation, dead-end filtration, and a final ultra-filtration yielding a high-quality, highly concentrated product at a recovery rate up to 92% (Tables 3.4 and 3.5).

Table 3.4 *H. polymorpha*-based products (selection)

	Product	Status	Brand name	Reference
Pharmaceutical	HBsAg (*adr*)	Launched	HepaVax Gene	Schaefer et al., 2002
	HbsAg (*adw*)	Launched		Schaefer et al., 2002
	insulin	Launched	AgB	
	IFNα -2a	Process transfer	Wosulin	Müller et al., 2001
	HSA	Pilot scale completed		Heo et al., 2003
	EGF	Lab scale completed		Heo et al., 2002
Food additive	Hexose oxidase	Launched	Grindamyl-Surebake	Cook and Thygesen, 2003
Feed additive	Phytase	Registration		Mayer et al., 1999
Enzymes	Levansucrase	Lab scale completed		Park et al., 2004

Table 3.5 Selection of *H. polymorpha* host strains

Strain	Genotype	Phenotype	Source
Parental strain			
DL-1	wild-type (NRRL-Y-7560,ATCC26012)		Levine and Cooney, 1973
DL-1-L	*leu2*	Leu^-	Sohn et al., 1996
uDL10	*leu2 ura3*	Leu^-Ura^-	KRIBB
DL1Δ-A	*leu2 Δade2*	Leu^-Ade^-	CRC
uDLB11	*leu2 ura3 Δpep4::lacZ*	$Leu^-Ura^-Pep4^-$	KRIBB
uDLB12	*leu2 ura3 Δprc1::lacZ*	$Leu^-Ura^-Prc1^-$	KRIBB
uDLB13	*leu2 ura3 Δkex1::lacZ*	$Leu^-Ura^-Kex1^-$	KRIBB
DLT2	*leu2 Δmox-trp3::ScLEU2*	Mox^-Trp^-	CRC
DL1-LΔM	*leu2 Δmox*	Leu^-Mox^-	CRC)
Parental strain			
NCYC495	wild-type (CBS1976, ATAA14754, NRRL-Y-1798, VKM-Y-1397)		Wickerham, 1951
L1	*leu1-1**	Leu^-	Gleeson et al., 1986
A11	*ade11-1*	Ade^-	Parpinello et al., 1998
M6	*met6-1*	Met^-	Parpinello et al., 1998
Nitrate assimilation-related strains			
NAG1995	Δ*ynr1::URA3*, *leu1-1**	Ynr1 Leu^-	Avila et al., 1995
NAG1996	Δ*yni1::URA3*, *leu1-1**	Yni1 Leu^-	Brito et al., 1996
NAG997	Δ*ynt1::URA3*, *leu1-1**	Ynt1 Leu^-	Pérez et al., 1997
NAG998	Δ*yna1::URA3*, *leu1-1**	Yna1 Leu^-	Avila et al., 1998

(continued)

Table 3.5 (continued)

Strain	Genotype	Phenotype	source
Parental strain			
CBS4732	wild-type (CCY38-22-2, ATCC34438, NRRL-Y-5445)		Morais and Maia, 1959
LR9/RB11/ MedHp1	*ura3-1*		Roggenkamp et al., 1986; Weydemann et al., 1995
RB11/MedHp2	*ura3 leu1-1*		Kang and Gellissen, 2005
RB14/MedHp3	*ura3 leu1-1, ade*		
A16	*leu2* *trp3 mox*	Leu$^-$ Trp$^-$Mox$^-$	Veale et al., 1992
1B	*ade2-88 leu2-2*	Ade$^-$Leu$^-$	Bogdanova et al., 1998
1-HP065	*ade2-88 ura2-1 met 4-220*	Ade$^-$Leu$^-$Met$^-$	Mannazzu et al., 1997
14C	*leu2-2 cat1-14*	Leu$^-$Cat$^-$	Lahtchev, 2002
5C-HP156	*ade2-88*	Ade$^-$	Lahtchev, 2002
8V	*leu2*	Leu$^-$	Agaphonov et al., 1995

* *leu1-1* and *leu2* correspond to the same gene.

3.7 Future Directions and Conclusions

Despite the most favorable characteristics of the *H. polymorpha*-based platform for application in heterologous gene expression, problems and limitations can be encountered in particular strain and product developments as is similarly and more frequently observed in other yeast systems. These limitations include overglycosylation (Agaphonov et al., 2001), retention within the ER (Agaphonov et al., 2002), poor secretion, impaired processing (Müller et al., 2002; Gellissen et al., 2002) and proteolytic degradation (Suckow and Gellissen, 2002). A possible strategy to overcome these limitations is to identify genes and gene products that may upon disruption or co-expression positively influence the performance of respective strains. As such, the *S. cerevisiae*-derived *KEX2* gene provided a greatly improved processing of a IFN-2a pre-pro-sequence in *H. polymorpha* in which production of predominantly N-terminally extended molecules had been observed before (Müller et al., 2001; Gellissen et al., 2002). In other examples co-expression of a *S. cerevisiae*-derived *CMK2* or the *H. polymorpha CNE1* (calnexin) gene has led to an improved secretion and a reduction in overglycosylation of a secreted enzyme and a cytokine as described before.

The tools of functional genomics established in the recent past will further the identification of genes hat can potentially ease limitations and drawback of

heterologous protein production in a given case. However, there is obviously no single yeast platform that is optimal for all proteins. It is, therefore, advisable to assess several yeast platforms in parallel for their capability to produce a particular protein in desired amounts and quality. This approach is now greatly facilitated by the newly developed wide-range yeast vector system (CoMed™) (Steinborn et al., 2006). The range of established yeast expression platforms are transformed simultaneously by an individual single vector, thereby enabling an assessment in parallel of such strains for criteria like efficient secretion or authentic processing and modification.

References

Agaphonov, M.O., Beburov, M.Y., Ter-Avanesyan, M.D., and Smirnov, V.N. 1995. *Yeast* **11:** 1241–1247.

Agaphonov, M., Trushkina, P.M., Sohn, J.S., Choi, E.S., Rhee, S.K., and Ter-Avanesyan, M.D. 1999. *Yeast* **15:** 541–551.

Agaphonov, M.O., Packeiser, A.N., Chechenova, M.B., Choi, E.-S., and Ter-Avanesyan, M.D. 2001. *Yeast* **18:** 391–402.

Agaphonov, M.O., Romanos, N.V., Trushina, P.M., Smirnov, V.N., and Ter-Avanesyan, M.D. 2002. BMC *Mol. Biol*. **3:** 15–22.

Amuel, C., Gellissen, G., Hollenberg, C.P., and Suckow, M. 2000. *Biotechnol. Bioproc. Eng*. **5:** 247–52.

Avgerinos, G.C., Turner, B.G., Gorelick, M.D., Papendieck, A., Weydemann, U., and Gellissen, G. 2001. *Sem. Thrombos. Hemostas*. **27:** 357–371.

Avila, J., González, C., Brito, N., and Siverio, J.M. 1998. *Biochem. J*. **335:** 647–652.

Avila, J., Pérez, M.D., Brito, N., González, C., and Siverio, J.M. 1995. *FEBS Lett*. **366:** 137–142.

Baerends, R.J.S., Sulter, G.J., Jeffries, T.W., Cregg, J.M., and Veenhuis, M. 2002. *Yeast* **19:** 37–42.

Barnett, J.A., Payne, R.W., and Yarrow, D. 2000. *Yeasts: Characteristics and identification*, 3rd edn., Cambridge University Press, Cambridge, UK.

Bartelsen, O., Barnes, C.S., and Gellissen, G. 2002. In: Gellissen G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley VCH, Weinheim, pp. 211–228.

Birney, E., and Durbin, R. 2000. *Genome Res*. **10:** 547–548.

Bogdanova, A.I., Kustikova, O.S., Agaphonov, M.O., and Ter-Avanesyan, M.D. 1998. *Yeast* **14:** 1–9.

Brake, A.J., Merryweather, J.P., Coit, D.G., Heberlein, U.A., Masiarz, F.R., Mullenbach, G.T., Ureda, M.S., Valenzuela, P., and Barr, P.J. 1984. Proc. Natl. Acad. Sci. USA **81:** 4642–4646.

Bretthauer, R.K., and Castellano, F.J. 1999. *Biotechnol. Appl. Biochem*. **30:** 193–200.

Brito, N., Avila, J., Pérez, M.D., González, C., and Siverio, J.M. 1996. *Biochem. J*. **317:** 89.–95

Brito, N., Pérez, M.D., Perdomo, G., González, C., García-Lugo, P., and Siverio, J.M. 1999. *Appl. Microbiol. Biotechnol*. **53:** 23–29.

Brocke, P., Schaefer, S., Melber, K., Jenzelewski, V., Müller, F., Dahlems, U., Bartelsen, O., Park, K.N., Janowicz, Z.A., and Gellissen, G. 2004. In: Production of recombinant proteins: Novel microbial and eukaryotic expression systems (ed. Gellissen, G.), Wiley VCN, Weinheim, pp. 319–60.

Callewaert, N., Laroy, W., Cadirgi, H., Geysens, S., Saelnes, X., Jou, W.M., and Contreras, R. 2001. *FEBS Lett*. **503:** 173–178.

Chiba, Y., Suzuki, M., Yoshida, S., Yoshida, A., Ikenaga, H., Takeuchi, M., Jigami, Y., and Ichishima, E. 1998. *J. Biol. Chem*. **273:** 26298–26304.

Cook, M.W., and Thygesen, H.V. 2003. Food Chem. *Toxicol*. **41:** 523–529.

Cox, H., Mead, D., Sudbery, P., Eland, M., and Evans, L. 2000. *Yeast* **16:** 1191–1203.

Degelmann, A., Müller, F., Sieber, H., Jenzelewski, V., Suckow, M., Strasser, A.W.M., and Gellissen, G. 2002. *FEMS Yeast Res*. **2:** 349–361.

Didion, T., and Roggenkamp, R. 1992. *FEBS Lett.* **303:** 113–116.

Gellissen, G. 2000. Appl. Microbiol. Biotechnol. **54:** 741–750.
Gellissen, G., 2002. *Hansenula polymorpha* - biology and applications. Wiley-VCH, Weinheim.
Gellissen, G. and Hollenberg, C.P. 1997. *Gene* **190:** 87–97.
Gellissen, G. and Veenhuis, M. 2001. *Yeast* **18:** 1–3.
Gellissen, G., Janowicz, Z.A., Weydemann, U., Melber, K., Strasser, A.W.M., and Hollenberg, C.P. 1992a. *Biotechnol. Adv*. **10:** 179–189.
Gellissen, G., Melber, K., Janowicz, S.A., Dahlems, U., Weydemann, U., Piontek, M., Strasser, A. W.M., and Hollenberg, C.P. 1992b. *Antonie van Leeuwenhoek* **62:** 79–93.
Gellissen, G., Müller, F., Sieber, H., Tieke, A., Jenzelewski, V., Degelmann, A., and Strasser, A.W.M. 2002. In: Gellissen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley VCH, Weinheim, pp. 229–254.
Gemmill, T.R. and Trimble, R.B. 1999. *Biochim. Biophys. Acta* **1426:** 227–237.
Gleeson, M.A., Ortori, G.S., and Sudbery, P.E. 1986. *J. Gen. Microbiol*. **132:** 3459–3465.
Gonzalez, C., Perdomo, G., Tejera, P., Brito, N., and Siverio, J.M. 1999. *Yeast* **15:** 1323–1329.
Guengerich, L., Kang, H.A., Behle, B., Gellissen, G., and Suckow, M. 2004. (In: Kück U. Ed.), *The mycota II. Genetics and Biotechnology* Springer-Verlag, Heidelberg, pp. 273–287.
Hamilton, S.R., Bobrowicz, P., Bobrowicz, B., Davidson, R.C., Li, H., Mitchell, T., Nett, J.H., Rausch, S., Stadheim, T.A., Wischenwski, H., Wildt, S., and Gerngross, T.U. 2003. *Science* **301:** 1244–46.
Hamilton, S.R., Davidson, R.C., Sethuraman, N., Nett, J.H., Jiang, Y., Rios, S., Bobrowicz, P., Stadheim, T.A., Li, H., Choi, B.-K., Hopkins, D., Wischenwski, H., Roser, J., Mitchell, T., Strawbridge, R.R., Hoopes, J., Wildt, S., and Gerngross, T.U. 2006. *Science* **313:** 1441–1443.
Hazeu, W., Bruyn, J.C., and de Bos, P. 1972. *Arch. Microbiol*. **87:** 185–188.
Hellwig, S., Stöckmann, C., Gellissen, G., and Büchs, G. 2005. In: *Production of recombinant proteins: novel microbial and eukaryotic expression systems* (Gellissen, G. Ed.), Wiley VCN, Weinheim, pp. 287–317.
Heo, J.H., Hong, W.K., Cho, E.Y., Kim, M.W., Kim, J.Y., Kim, C.H., Rhee, S.K., and Kang, H.A. 2003. *FEMS Yeast Res*. **4:** 175–184.
Heo, J.H., Won, H.S., Kang, H.A., Rhee, S.K., and Chung, B.H. 2002. *Protein Expr. Purif*. **24:** 117–122.
Hollenberg, C.P. and Janowicz, Z.A. 1988. EPA 0299108.
Janowicz, Z.A., Eckart, M.R., Drewke, C., Roggenkamp, R.O., Hollenberg, C.P., Maat, J., Ledeboer, A.M., Visser, C., and Verrips, C.T. 1985. *Nucleic Acids Res*. **13:** 3043–3306.
Jenzelewski, V. 2002. In: Gellissen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley VCH, Weinheim, pp. 156–174.
Jigami, Y. and Odani, T. 1999. *Biochim. Biophys*. *Acta* **1426:** 335–345.
Kang, H.A. and Gellissen G. 2005. In: Gellissen, G. (Ed.), *Production of recombinant proteins: novel microbial and eukaryotic expression systems,* Wiley VCN, Weinheim, pp. 111–142.
Kang, H.A., Kim, J.-Y., Ko, S.-M., Park, C.S., Ryu, D.Y., Sohn, J.-H., Choi, E.-S., and Rhee, S.-K. 1998a. *Yeast* **14:** 1233–1240.
Kang, H.A., Sohn, J.-H., Choi, E.-S., Chung, B.-H., Yu, M.-H., and Rhee, S.-K. 1998b. *Yeast* **14:** 371–381.
Kang, H.A., Kang, W., Hong, W.-K., Kim, M.W., Kim, J.-Y., Sohn, J.-H., Choi, E.-S., Choe, K.-B., and Rhee, S.-K. 2001a. *Biotechnol. Bioeng*. **76:** 175–185.
Kang, H.A., Hong, W.-K., Sohn, J.-H., Choi, E.-S., and Rhee, S.K. 2001b. *Appl. Microbiol. Biotechnol.* **55:** 734–741.
Kang, H.A., Sohn, J.-H., Agaphonov, M.O., Choi, E.-S., Ter-Avanesyan, M.D., and Rhee, S.K. 2002. *Hansenula polymorpha* - biology and applications, Wiley VCH, Weinheim, pp. 124–146.
Kim, M.W., Agaphonov, M.O., Kim, J.-Y., Rhee, S.K., and Kang, H.A. 2002. Yeast 19: 863–871.
Kim, M.W., Rhee, S.K., Kim, J.Y., Shimma, Y.I., Chiba, Y., Jigami, Y., and Kang, H.A. 2004. *Glycobiology* **14:** 243–251.
Kim, M.W., Kim, E.J., Kim, J.-Y., Park, J.-S., Oh, D.-B., Shimma, J.-I., Chiba, Y., Jigami, Y., Rhee, S.K., and Kang, H.A. 2006. *J. Biol. Chem*. **281:** 6261–6272.

Kim, S.Y., Sohn, S.-H., Kang, H.A., and Choi, E.-S. 2001. *Yeast* **18:** 455–461.
Kim, S.Y., Sohn, J.H., Pyun, Y.R., and Choi, E.S. 2002. *Yeast* **19:** 1153–1163.
Kim, S.Y., Sohn, J.H., Bae, J.H., Pyun, Y.R., Agaphonov, M.O., and Ter-Avanesyan, M.D., Choi E.S. 2003. Appl. Environ. *Microbiol*. **69:** 4448–4454.
Klabunde, J., Diesel, A., Waschk, D., Gellissen, G., Hollenberg, C.P., and Suckow, M. 2002. *Appl. Microbiol. Biotechnol*. **58:** 797–805.
Klabunde, J., Kunze, G., Gellissen, G., and Hollenberg, C.P. 2003. *FEMS Yeast Res.* **4:** 185–193.
Kurtzman, C.P. 1984. *Antonie van Leeuwenhoek* **50:** 209–217.
Kurtzman, C.P. and Fell J.W. 1998. The yeasts, a taxonomic study, Elsevier, Amsterdam, 4th edn.
Kurtzman, C.P. and Robnett C.J. 1998. *Antonie van Leeuwenhoek* **73:** 331–371.
Lahtchev, K. 2002. (Gellisen, G. Ed.), Basic genetics of *Hansenula Polymorpha.* In: *biology and applications*, Wiley VCH, Weinheim, pp. 8–20.
Ledeboer, A.M., Edens, L., Maat, J., Visser, C., Bos, J.W., Verrips, C.T., Janowicz, Z.A., Eckart, M., Roggenkamp, R.O., and Hollenberg, C.P. 1985. *Nucleic Acids Res*. **13:** 3063–3082.
Levine, D.W. and Cooney, C.L. 1973. *Appl. Microbiol*. **26:** 982–989.
Mannazzu, I., Gierra, E., Strabbioli, R., Masia, A., Maestrale, G.B., Zoroddu, M.A., and Fatichenti, F. 1997. *FEMS Microbiol. Lett*. **147:** 23–28.
Mayer, A.F., Hellmuth, K., Schlieker, H., Lopez-Ulibarri, R., Oertel, S., Dahlems, U., Strasser, A.W.M., van Loon, A.P.G.M. 1999. *Biotechnol. Bioeng*. **63:** 373–381.
Middelhoven, W. 2002. (Gellissen, G. Ed.), In: *Hansenula polymorpha - biology and applications* Wiley VCH, Weinheim, pp. 1–7.
Montesino, R., García, R., Quintero, O., and Cremata, J.A. 1998. *Protein Exp. Purif.* 14: 197–207.
Morais, J.O.F. and de Maia M.H.D. 1959. *Anais de Escola Superior de Quimica de Universidade do Recife* **1:** 15–20.
Müller, S., Sandal, T., Kamp-Hansen, P., and Dalbøge, H. 1998. *Yeast* **14:** 1267–1283.
Müller, F. II, Tieke, A., Waschk, D., Mühle, C., Müller, F. I, Seigelchifer, M., Pesce, A., Jenzelewski, V., and Gellissen, G. 2002. *Proc. Biochem*. **38:** 15–25.
Ogata, K., Nishikawa, H., and Ohsugi, M. 1969. *Agric. Biol. Chem*. **33:** 1519–1520.
Oh, K.S., Kwon, O., Oh, Y.W., Sohn, M.J., Jung, S., Kim, Y.K, Kim, M.G., Rhee, S.K., Gellissen, G., and Kang, H.A. 2004. *J. Microbiol. Biotechnol*. **14:** 1239–1248.
Osoegawa, K., de Jong, P.J., Frengen, E., and Ioannou, P.A. 1999. *Current Protocols in Human Genetics*, 5.15.1–5.15.33.
Osoegawa, K., Woon, P.Y., Zhao, B., Frengen, E., Tateno, M., Catanese, J.J., de Jong, P.J. 1998. *Genomics* **52:** 1–8.
Papendieck, A., Dahlems, U., and Gellissen, G. 2002. In: Gelllissen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley-VCH, Weinheim, pp. 255–271.
Park, B.S., Ananine, V., Kim, C.H., Rhee, S.K., and Kang, H.A. 2004. *Enz. Microb. Technol*. **34:** 132–138.
Park, J.-N., Sohn, M.J., Oh, D.-B., Kwon, O., Rhee, S.K., Hur, C.-G., Lee, S.Y., Gellissen, G., and Kang, H.A. 2007. *Appl. Environm. Microbiol*. **73:** 5990–6000.
Parpinello G., Berardi E., and Strabbioli R. 1998. *J. Bacteriol*. **180:** 2958–2967.
Perez M.D., Gonzalez C., Avila J., Brito N., Siverio J.M. 1997. *Biochem. J.* **15:** 397–403.
Phongdara, A., Merckelbach, A., Keup, P., Gellissen, G., and Hollenberg, C.P. 1998. *Appl. Microbiol. Biotechnol*. **50:** 77–84.
Ramezani-Rad, M., Hollenberg, C.P., Lauber, J., Wedler, H., Griess, E., Wagner, C., Albermann , K., Hani, J., Piontek, M., Dahlems, U., and Gellissen, G. 2003. *FEMS Yeast Res*. **4:** 207–215.
Reinders, A., Romano, I., Wiemken, A., and de Virgilio, C. 1999. *J. Bacteriol*. **181:** 4665–4668.
Roggenkamp, R., Hansen, H., Eckart, M., Janowicz, Z.A., and Hollenberg, C.P. 1986. *Mol. Gen. Genet.* **202:** 302–308.
Romano, I. 1998. Ph.D. Thesis, University of Basel, Switzerland.
Schaefer, S., Piontek, M., Ahn, S.-J., Papendieck, A., Janowicz, S.A., and Gellissen, G. 2001 (Dembowsky, K., and Stadler, P. Eds.). In: *Novel therapeutic proteins: selected case studies*, Wiley-VCN, Weinheim, pp. 245–274.

Schaefer, S., Piontek, M., Ahn, S.-J., Papendieck, A., Janowicz, Z.A., Timmermans, I., Gellissen, G. 2002. In: Gellissen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley-VCH, Weinheim, pp. 175–210.

Siverio, J.M. 2002. In: Gellissen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley VCH, Weinheim, pp. 21–40.

Sohn, J.-H., Choi, E.-S., Kim, C.-H., Agaphonov, M.O., Ter-Avanesyan, M.D., and Rhee, J.-S., and Rhee, S.-K. 1996. *J. Bacteriol*. **178:** 4420–4428.

Sohn, J.-H., Choi, E.-S., Kang, H.A., Rhee, J.-S., and Rhee, S.-K. 1999. J. Bacteriol. 181: 1005–1013.

Steinborn, G., Gellissen, G., and Kunze, G. 2005. *FEMS Yeast Res*. **5:** 1047–1054.

Steinborn, G., Böer, E., Scholz, A., Tag K., Kunze G., and Gellissen, G. 2006. *Microbial Cell Factories* **5:** 33.

Suckow, M. and Gellissen, G. 2002. In: Gellissen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley VCH, Weinheim, pp. 105–123.

Sudbery, P. 2003. *Yeast* **20:** 1307–1308.

Tani, Y. 1984. In: Hou, C.T. (Ed.), *Methylotrophs: microbiology, biochemistry, and genetics*, CRC Press, Boca Raton, FL, pp. 55–86.

Teunisson, D.J., Hall, H.H., and Wickerham, L.J. 1960. *Mycologia* **52:** 184–188.

Tikhomirova, L.P., Ikonomova, R.N., Kuznetsova, E.N., Fodor, I.I., Bystrykh, L.V., Aminova, L.R., and Trotsenko, Y.A. 1988. *J. Basic Microbiol*. **28:** 343–351.

van Dijk, R., Faber, K.N., Hammond, A.T., Glick, B.S., Veenhuis, M., and Kiel, J.A.K.W. 2001. *Mol. Genet. Genomics* **266:** 646–656.

van Djik, R., Faber, K.N., Kiel, J.A.K.W., Veenhuis, M., and van der Klei, I.J. 2000. *Enzyme Microb. Technol*. **26:** 793–800.

van der Klei, I.J., and Veenhuis, M. 2002. In: Gellissen, G. (Ed.), *Hansenula polymorpha - biology and applications* Wiley VCH, Weinheim, pp. 76–94.

Veale R.A., Guiseppin M.L., van Eijk H.M., Sudbery P.E., and Verrips C.T. 1992. *Yeast* **8:** 361–372.

Weydemann, U., Keup, P., Piontek, M., Strasser, A.W.M., Schweden J., Gellissen G., and Janowicz Z.A. 1995. *Appl. Microbiol. Biotechnol*. **44:** 377–385.

Wickerham, L.J. 1951. *Technical bulletin No 1029*, US Dept Agric, Washington DC, pp. 1–56.

Yurimoto H., Sakai Y., and Kato N. 2002. In: Gellisen, G. (Ed.), *Hansenula polymorpha - biology and applications*, Wiley VCH, Weinheim, pp. 61–75.