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MiniReview

New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica* – A comparison

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Abstract

Yeasts combine the ease of genetic manipulation and fermentation of a microbial organism with the capability to secrete and to modify proteins according to a general eukaryotic scheme. Yeasts thus provide attractive platforms for the production of recombinant proteins. Here, four important species are presented and compared: the methylotrophic *Hansenula polymorpha* and *Pichia pastoris*, distinguished by an increasingly large track record as industrial platforms, and the dimorphic species *Arxula adeninivorans* and *Yarrowia lipolytica*, not yet established as industrial platforms, but demonstrating promising technological potential, as discussed in this article.

Keywords: *Hansenula polymorpha*; *Pichia pastoris*; *Arxula adeninivorans*; *Yarrowia lipolytica*; Yeast expression platforms

1. Introduction

The exploitation of recombinant-DNA technology to engineer expression systems for heterologous protein production has provided a major task during the last decades. Production procedures had to be developed that employ platforms which meet both the demand for efficient mass production and criteria of safety and authenticity of the produced compounds [1,2]. In this respect, yeasts offer considerable advantages over alternative microbial and eukaryotic cellular systems in providing low-cost screening and production systems for authentically processed and modified compounds. The four selected organisms furthermore meet safety prerequisites in that they do not harbour pyrogens, pathogens or viral inclusions.

The initial yeast system developed for heterologous gene expression was based on the baker's yeast *Saccharomyces cerevisiae*. This platform has been successfully applied to the production of various FDA-approved pharmaceuticals, including insulin [3] and HBsAg [4]. However, when using this system, certain limitations

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and drawbacks are often encountered, since *S. cerevisiae* tends to hyperglycosylate recombinant proteins; N-linked carbohydrate chains are terminated by mannose attached to the chain via an α1,3 bond, which is considered to be allergenic. Other restrictions are the consequence of the limited variety of carbon sources that can be utilised by this species, which limits the fermentation design options. Sometimes, the preferential use of episomal vectors leads to instabilities of recombinant strains; as a result, batch inconsistencies of production runs can be of major concern [2].

Therefore alternative yeast systems have been defined that can potentially overcome the described limitations of the traditional baker's yeast. The following comparison includes four platforms, chosen as examples out of a wide range of yeast-based systems now available: the two methylotrophic yeast species *Hansenula polymorpha* (*H.p.*) [5,6] and *Pichia pastoris* (*P.p.*) [5,7], and the two dimorphic organisms *Arxula adeninivorans* (*A.a.*) [8,9] and *Yarrowia lipolytica* (*Y.l.*) [10,11]. The selected organisms share the capability to utilize a broad range of carbon sources, two of them (*H.p.* and *A.a.*) can assimilate nitrate, all but *P.p.* are thermo-tolerant species with *A.a.* and *Y.l.* exhibiting a temperature-dependent dimorphism with hyphae formed at elevated temperature. For all selected systems a range of host strains and relevant genetic elements is available.

In case of the two methylotrophic species, engineered strains have been developed that exhibit human-like *N*-glycosylation patterns (H.-A. Kang, pers. commun.) [12]. The genome of both species has been completely deciphered (J.M. Cregg, pers. commun.) and for *H.p.* a microarray chip is available [13,14]. Accordingly both species are distinguished by an expanding track record as production hosts for industrial and pharmaceutical proteins, for both systems efficient secretion of recombinant proteins (>10 g l^{-1}) have been reported [15,16]. All of this attests to the favourable characteristics of these two yeast species. In the case of the two dimorphic species, data on chromosomes and on a partial characterization of the genomes are already available [9,17–19]. The dimorphic yeasts *A. adeninivorans* and *Y. lipolytica* represent more recently defined systems that have yet to establish themselves but that demonstrate a promising potential for industrial processes. A more detailed description of the four platforms can be found in a recent book on production of recombinant proteins and various chapters therein [1,2,6,7,9,11].

Despite the superiority of some characteristics in the examples described below, there is clearly no single system that is optimal for the production of all possible proteins. Predictions of a successful development for a given protein can only be made to a certain extent when restricting the initial strain engineering to a single species. The availability of a wide-range yeast vector system enables the assessment of several yeasts in parallel for their capability to produce a particular protein in desired amounts and quality [20–22]. Applied to the four selected, divergent species a high probability of success can be envisaged.

2. History, phylogenetic position, basic genetics and biochemistry of the four selected yeast species

2.1. H. polymorpha *and* P. pastoris

A limited number of yeast species is able to utilize methanol as sole energy and carbon source. These include *H. polymorpha*, *P. pastoris*, *Candida boidinii* and *P. methanolica* [5]. The first methylotrophic yeast described was *Kloeckera* sp. No. 2201, later re-identified as *C. boidinii* [23]. Subsequently other species, including *H. polymorpha* and *P. pastoris*, were identified as having methanol-assimilating capabilities [24]. The taxonomic position of the two species is shown in Fig. 1.

P. pastoris was initially chosen for the production of single-cell proteins (SCP) for feedstock due to its efficient growth in methanol-containing media, since synthesis of methanol from natural methane was inexpensive in the late 60s. However, as the costs of methane increased in the oil crisis in the 1970s, and that of soy beans, the major feedstock source, decreased, production of SCP in this yeast never became economically competitive [25,26]. With the methods of recombinant DNA emerging it was developed as an efficient system for heterologous gene expression instead, using for the main part elements derived from methanol-metabolic pathway genes [25,26]. *P. pastoris* normally exists in the vegetative haploid state with vegetative, multilateral budding. Nitrogen limitation results in mating and the formation of diploid cells. The organism is

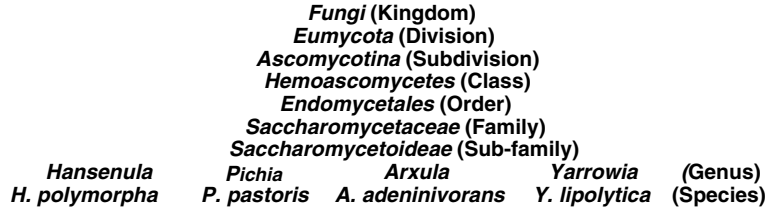

Fig. 1. Taxonomy of the four selected yeasts (after Barnett et al. [40]).

considered to be homothallic. Diploid cells cultured in a standard vegetative medium remain diploid. When transferred to nitrogen-limited conditions, they undergo meiosis and produce haploid cells. Since it is most stable in its vegetative haploid state, easy isolation and characterization of mutants is possible. All *P. pastoris* expression strains are derived from strain NRRL-Y 11430. A selection of these strains is provided later.

In case of *H. polymorpha* three basic strains with unclear relationships, different features, and independent origins are used in basic research and biotechnological application: strain CBS4732 (CCY38-22-2; ATCC34438, NRRL-Y-5445) was initially isolated by Morais and Maia [27] from soil irrigated with waste water from a distillery in Pernambuco, Brazil. Strain DL-1 (NRRL-Y-7560; ATCC26012) was isolated from soil by Levine and Cooney [26]. The strain named NCYC495 (CBS1976; ATAA14754, NRLL-Y-1798) is identical to a strain first isolated by Wickerham [28] from spoiled concentrated orange juice in Florida and initially designated *H. angusta*. Strains CBS4732 and NCYY495 can be mated whereas strain DL-1 cannot be mated with the other two (K. Lahtchev, pers. commun.).

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 per ascus. Ascigenic cells are diploid, arising from conjugation of haploid cells. The genus is predominantly heterothallic. *H. polymorpha* is probably homothallic, exhibiting an easy interconversion between the haploid and diploid state [30,31]. Like *P. pastoris* it has been developed as expression platform using elements that include strong inducible promoters derived from genes of the methanol utilization pathway [32].

Since *H. polymorpha* is the more thermo-tolerant of the two it might be better suited as source and for the production of proteins considered for crystallographic studies. In basic research it is used as model organism for research on peroxisomal function and biogenesis [33,34], as well as nitrate assimilation [35]. The presence of a nitrate assimilation pathway is a feature not shared by *P. pastoris*.

Methylotrophic yeast species share a compartmentalized methanol-metabolic pathway which has been detailed elsewhere [36] (Fig. 2). During growth on methanol key enzymes of this metabolism are present in high amounts and peroxisomes proliferate (Fig. 3). An especially high abundance can be observed for AOX (alcohol oxidase), FMD (formate dehydrogenase), and DHAS (dihydroxyacetone synthase) [37]. Their synthesis is regulated at the transcriptional level of the respective genes. In *P. pastoris* two *AOX* genes are present. Gene expression is subject to a carbon source-dependent repression/derepression/induction mechanism conferred by inherent properties of their promoters. Promoters are repressed by glucose, derepressed by glycerol, and induced by methanol. These promoter elements, in particular the elements derived from the *MOX* (*AOX1* in *P. pastoris*) and the *FMD* genes, constitute attractive components for the control of heterologous gene expression that can be regulated by carbon source addition to a medium. The possibility of eliciting high promoter activity with glycerol as sole carbon source and even with limited addition of glucose (glucose starvation) in *H. polymorpha* is unique among the methylotrophic yeasts. In *P. pastoris* the active status of the

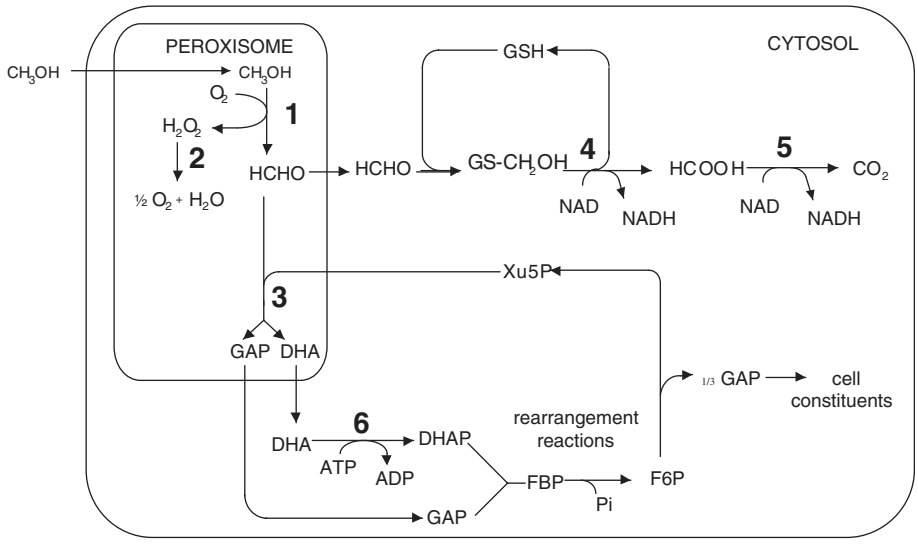

Fig. 2. Methanol metabolism pathway in methylotrophic yeasts. 1 – alcohol oxidase, 2 – catalase, 3 – dihydroxyacetone synthase, 4 – formaldehyde dehydrogenase, 5 – formate dehydrogenase, 6 – dihydroxyacetone kinase, GSH – glutathione, Xu5P – xylulose-5-phosphate, FBP – fructose-1,6-bisphosphate.

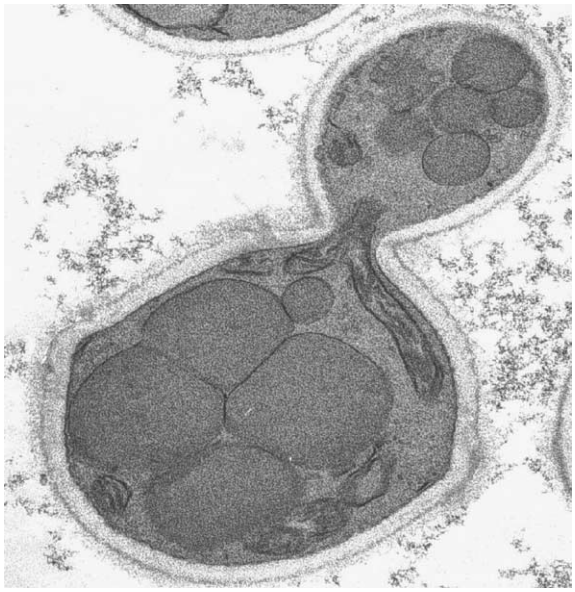

Fig. 3. Micrograph of a budding *H. polymorpha* cell, grown in a chemostat under methanol conditions.

promoter is strictly dependent on the presence of methanol or methanol derivatives [5,6]. However, this does not seem to be an inherent promoter characteristic but it rather depends on the cellular environment of the specific host, as upon transfer into *H. polymorpha* the *P. pastoris*-derived *AOX1* promoter is active under glycerol conditions [38,39].

2.2. A. adeninivorans *and* Y. lipolytica

In 1984 Middelhoven et al. [41] described a yeast species isolated from soil by enrichment culturing, named *Trichosporon adeninovorans*. The type strain CBS 8244T displayed unusual biochemical activities. It was shown that it was able to assimilate a range of amines, adenine and several other purine compounds as sole energy and carbon source.

A second strain, LS3 (PAR-4) was isolated in Siberia (Y.G. Kapultsevich, Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia) from wood hydrolysates. As in the first case this strain was found to use a very large spectrum of substances as carbon and nitrogen sources [42].

In 1990, three additional *Tr. adeninovorans* strains were isolated from chopped maize herbage ensiled at 25 or 30 °C in The Netherlands, and yet another four strains were detected in humus-rich soil in South Africa [43]. A new genus name *Arxula* Van der Walt, M.T. Smith and Yamada (*Candidaceae*) was proposed for all these strains, which share properties like nitrate assimilation and xerotolerance. All representatives of the new proposed genus were ascomycetes, anamorphic and arthroconidial [43]. The genus *Arxula* comprises two species, the type species of the genus *A. terrestre* (Van der Walt and Johanssen) Van der Walt, M.T. Smith and Yamada, nov. comb., and *A. adeninivorans* (Middelhoven, Hoogkamer te-Niet and Kreger-van Rij) Van der Walt, M.T. Smith and Yamada, nov. comb.

A detailed physiological description of the yeast was provided by Gienow et al. [42] and Middelhoven et al. [44,45]. *A. adeninivorans* is able to assimilate nitrate like *H. polymorpha*. It can utilize a range of compounds as sole energy and carbon source including adenine, uric acid, butylamine, pentylamine, putrescine, soluble starch, melibiose, melezitose, propylamine or hexylamine [41]. It rapidly assimilates all the sugars, polyalcohols and organic acids used in the conventional carbon compound assimilation tests, except for L-rhamnose, inulin, lactose, lactate and methanol. Likewise, all conventionally used nitrogen compounds are suitable nitrogen sources with the exception of creatine and creatinine. Several nitrogen compounds, like amino acids and purine derivatives, are metabolized as sole carbon, nitrogen and energy source. This is also the case for many primary *n*-alkylamines and terminal diamines. In case of alcohols, dialcohols, carboxylic acids, dicarboxylic acids and other nitrogen-less analogous compounds, intermediates of the general metabolism are also assimilated. Furthermore, *A. adeninivorans* degrades some phenols and hydroxybenzoates.

Special features of biotechnological impact are the thermo-tolerance and temperature-dependent dimorphism which is especially pronounced in the strain LS3. This strain can grow at temperatures up to 48 °C without previous adaptation to elevated temperatures and is able to survive some hours at 55 °C [46]. Strain LS3 exhibits a temperature-dependent morphological dimorphism. Temperatures above 42 °C induce a reversible transition from budding cells to mycelial forms. Budding is re-established when the cultivation temperature is decreased below 42 °C. Wartmann et al. [47] selected mutants with altered dimorphism characteristics. These mutants grow already at 30 °C as mycelia. Cell morphology was found to influence the post-translational modifications of the Afet3p component of the iron transport system, an observation of potential impact for heterologous gene expression. *O*-glycosylation was found in budding cells only, whereas *N*-glycosylation occurred in both cell types. The characteristic of differential *O*-glycosylation may provide an option to produce heterologous proteins in both *O*-glycosylated and non-*O*-glycosylated form and to compare the impact of its presence on properties like biological activity or immunological tolerance [48]. A further interesting property of *A. adeninivorans* is its osmotolerance. It can grow in minimal as well as in rich media containing up to 3.32 osmomol/kg water in presence of ionic (NaCl), osmotic (PEG400) and water stress (ethylene glycol).

The hemiascomycetous yeast *Y. lipolytica* has been formerly known as *Candida*, *Endomycopsis* or *Saccharomycopsis lipolytica*. Its former classification in the *Candida* genus was due to the ignorance of its sexual cycle. The perfect form of this yeast was identified later on, in the late 1960s [49]. *Y. lipolytica* is the only known species in its genus, but the asexual taxon *C. neoformans* appears phylogenetically closely related [50]. Nearly all natural isolates of *Y. lipolytica* are haploid, heterothallic and belong either to the A or B mating type. Crossing of A and B mating types results in the formation of a stable diploid, which can be induced to sporulate, forming 1–4 spores per ascus.

Y. lipolytica metabolizes only few sugars (mainly glucose, but not sucrose), alcohols, acetate and hydrophobic substrates (such as alkanes, fatty acids and oils), as reviewed in [51]. The species is strictly aerobic, unlike most other hemiascomycetous yeasts. *Y. lipolytica* is considered as non-pathogenic, since the maximal growth temperature of most isolates does not exceed 32–34 °C. Moreover, several processes based on this yeast, mainly for the agro-food industry, have been classified as GRAS (generally regarded as safe) by the FDA (Food and Drug Agency). *Y. lipolytica* is a dimorphic organism like *A. adeninivorans* described before. Depending on the growth conditions it is able to form either yeast cells or hyphae and pseudohyphae.

Y. lipolytica diverges greatly from other ascomycetous yeasts by: (i) high GC content, (ii) high frequency of introns (13% of the genes have one or occasionally more introns), often of a relatively large size [52] (iii) unusual structure of its rDNA genes, (iv) low level of similarity of its genes with their counterparts in other yeasts (typically 50–60% at amino acid level), (v) unusual types of transposable elements of the LINE or MULE family [53], and others [53,54]. In evolutionary trees based on sequences of ''house-keeping'' genes, *Y. lipolytica* appears isolated from *Schizosaccharomyces pombe* on one hand, and from the group of other ascomycetous yeast species on the other [55,56].

3. The expression platforms based on the four selected yeasts

3.1. H. polymorpha *and* P. pastoris

For both methylotrophic species a range of host strains and integration/expression vectors exists that for the most part employ promoter elements derived from methanol utilization pathway genes [6,7] (Tables 1 and 2; Fig. 4 CoMed® vector).

In *H. polymorpha*, host strains are either derived from a CBS4732 (MedHp and RB series) or a ATCC26012 (DL-1 series) background; the plasmids applied for transformation are incorporated by homologous recombination and usually result in recombinant strains harbouring multiple tandemly repeated copies integrated into the target locus. For this purpose, plasmids harbouring one of a set of several cloned sub-telomeric *ARS* sequences derived from the DL-1 strain have been described [57,58]. A set of vectors has been used to target the heterologous DNA to the rDNA locus of *H. polymorpha* [20,59,60]. The presence of such a conserved rDNA integration segment renders the respective plasmids suited for transformation of a wide range of yeast species (see also article by Steinborn et al. [145] in this issue and the forthcoming Section 3 on the CoMed® vector/strain system). Furthermore co-transformation and co-integration of several plasmids is possible. Generally, circular plasmids harbouring a *HARS1* (*Hansenula ARS1*) sequence as a replication signal are used for recombinant strain generation. Subjection of the transformants to prolonged periods under selective conditions forces the genomic integration of the initially episomal plasmid which can be present in copy numbers of up to sixty in a head-to-tail cluster. The former assumption of a ''random integration'' of such plasmids is probably incorrect, since in a particular strain development for the production of a hepatitis B vaccine a recombination within the *FMD* locus was observed [6].

Table 1
ARS, rDNA regions, selection markers and promoter elements of the CoMed® vector system

Region/gene	Donor organism	Reference
ARS		
2 μm DNA	*S. cerevisiae*	[142]
ARS1	*S. cerevisiae*	[143]
HARS	*H. polymorpha*	[6]
SwARS	*Schw. Occidentalis*	[144]
rDNA region		
NTS2-ETS-18SrDNA-ITS1	*H. polymorpha*	[7]
25S rDNA	*A. adeninivorans*	[93]
18S rDNA	*A. adeninivorans*	[145]
ITS-5S-ETS-18S-ITS-5,8S-ITS	*A. adeninivorans*	[145]
NTS2-ETS-18SrDNA-ITS1	*A. adeninivorans*	[145]
Selection marker		
URA3	*S. cerevisiae*	[146]
LEU2	*S. cerevisiae*	[147]
ALEU2m	*A. adeninivorans*	[95]
ATRP1m	*A. adeninivorans*	[145]
HIS4	*P. pastoris*	[148]
Expression cassette (promoter)		
FMD promoter	*H. polymorpha*	[5]
MOX promoter	*H. polymorpha*	[5]
TPS1 promoter	*H. polymorpha*	[83]
AOX1 promoter	*P. pastoris*	[149]
TEF1 promoter	*A. adeninivorans*	[150]
AHSB4m promoter	*A. adeninivorans*	[122]
GAA promoter	*A. adeninivorans*	[151]
ALIP promoter	*A. adeninivorans*	[152]
AINV promoter	*A. adeninivorans*	[153]
AXDH promoter	*A. adeninivorans*	[154]
RPS7 promoter	*Y. lipolytica*	[106]

Table 2a
Selection of host strains for heterologous protein production

Strain	Genotype	Phenotype	Reference
Hansenula polymorpha[a]			
Parental strain DL-1 (NRRL-Y-7560, ATCC26012)			[28]
DL-1-L		Leu$^-$	[70]
DL10		Leu$^-$, Ura$^-$	[6]
uDLB11		Leu$^-$, Ura$^-$, Pep4$^-$	[6]
Parental strain NCYC495 (CBS1976, ATAA14754, NRRL-Y-1798, VKM-Y-1379)			[29]
L1		Leu$^-$	[155]
A11		Ade$^-$	[156]
NAG1996		Yni1$^-$, Leu$^-$	[157]
Parental strain CBS4732 (CCY-22-2, ATCC34438, NRRL-Y-5445)			[27]
LR9		Ura$^-$	[158]
MedHP1		Ura$^-$	Unpublished
MedHP2		Leu$^-$	Unpublished
Pichia pastoris[a]			
Parental strain NRRL-Y11430			[7]
GS115		Mut$^+$, His$^-$	[61]
SMD1168		Mut$^+$, His$^-$, Pep4$^-$	[26]
JC220		Ade$^-$, Arg$^-$, His$^-$, Ura$^-$	[159]
Arxula adeninivorans[a]			
Parental strain LS3 (SBUG724, PAR-4, MedAA-1)			[160]
135 (MedAA-2)		Adm$^-$ (mycelia at 30 °C)	[161]
R280-3		Lys$^-$, Pro$^-$, Leu$^-$	[162]
Yarrowia lipolytica[a]			
Po1d	*MatA*, *leu2-270*, *ura3-302*, *xpr2-322*	Leu$^-$, Ura$^-$, ΔAEP, Suc$^+$	[96]
Po1f	*MatA*, *leu2-270*, *ura3-302*, *xpr2-322*, *axp1*	Leu$^-$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	[111]
Po1g	*MatA*, *leu2-270*, *ura3-302::URA3*, *xpr2-322*, *axp1*	Leu$^-$, ΔAEP, ΔAXP, Suc$^+$, pBR322	[111]
Po1h	*MatA*, *ura3-302*, *xpr2-322*, *axp1-2*	Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	[5]

[a] Additionally strains of the CoMed® system as described in the text.

All *P. pastoris* strains applied for heterologous gene expressions are descendents of strain NRRL-Y-11430. Although autonomous episomal multicopy vectors exist for *P. pastoris* [61], strains were observed to be of higher productivity when expressing a recombinant gene from a single integrated copy instead from multicopy episomal vectors. Therefore recombinant strains are generated using integration approaches similar to those applied in *H. polymorpha*. The simplest way for integration into the genome is to linearize a vector at a unique site in either the marker gene (e.g., *HIS4*) or the *AOX1* promoter fragment and then use the linearized plasmid to transform an appropriate auxotrophic mutant. The free DNA termini stimulate homologous recombination events that result in single crossover-type integration events into these loci at high frequencies (50–80% of His$^+$ transformants) [61]. Alternatively, certain *P. pastoris* expression vectors can be digested in such a way that the expression cassette and marker gene are released, flanked by 5′ and 3′ *AOX1* sequences (see for example [62,63]). Approximately 10–20% of transformation events are the result of a gene replacement event in which the *AOX1* gene is deleted and replaced by the expression cassette and marker gene. The disruption of the *AOX1* gene forces these strains to rely on the *AOX2* gene for growth on methanol [62,64]. Since *AOX2* exhibits a lower expression profile, these strains have a Mut^s phenotype and can easily be selected by their reduced ability to grow on methanol.

Most *P. pastoris* transformants contain a single copy of an expression vector. Common approaches to construct multicopy expression strains in *P. pastoris* either employ transformation vectors harbouring multiple head-to-tail copies of an expression cassette [65] or expression vectors that contain a drug resistance gene as selection marker, like the bacterial *Kan^R*, *Zeo^R*, *Bsd^R* genes or the *P. pastoris FLD1* gene. With each of these genes, the level of drug resistance roughly correlates to

Table 2b
Selection of expression vectors

Plasmid	Selection marker	Features	Reference
H. polymorpha[a]			
AMIpL1	*LEU2*	Multiple cloning site, *HARS36* selection marker	[69]
pGLG61	*LEU2, Kan*^{r}	*Not*I/*Bam*HI sites, *TEL*188	[57]
pFPMT121	*URA3*	*Eco*RI, *Bgl*II, *Bam*HI sites, *HARS1*, *FMD*-promoter	[67]
P. pastoris[a]			
pPIC3.5K	*HIS4*, *Kan*^{R}	Multiple cloning site, G418 selection	Invitrogen
pPICZ	ble^R	Multiple cloning site, Zeocin selection	Invitrogen
pBLHIS-SX	*HIS4*	Series of vectors with P_{AOX1} fused to *MFα 1* pre-pro	[159]
A. adeninivorans[a]			
I1-X6	*LYS2*	*AILV* promoter	[92]
pAL-HPH1	*Hph*	Hygromycin selection	[93]
pAL-ALEU2m	*LEU2*		[93]
Y. lipolytica[a]			
pINA1269	*LEU*	Monocopy integration	[111]
p65IP	*URA3* (*ura3d1*)	rDNA integration	[109]
p64IP	*URA3* (*ura3d4*)	Multicopy rDNA integration	[109]
pINA1311	*URA3* (*ura3d1*)	Zeta sequence, monocopy auto-cloning	[112]

[a] Additionally plasmids and components of CoMed® system as described in text and in Table 1.

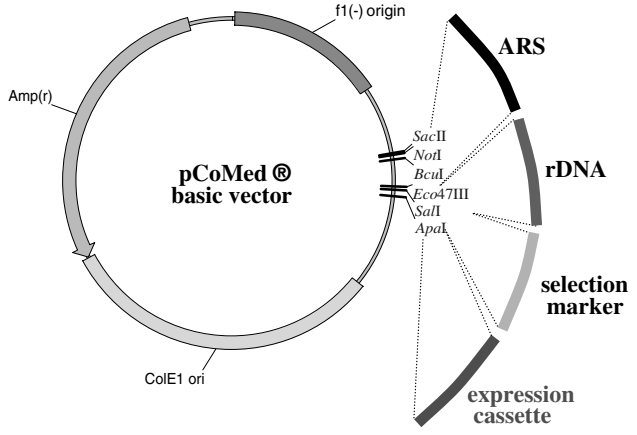

Fig. 4. Design and functionality of the CoMed® vector system. The CoMed® basic vector contains all *E. coli* elements for propagation in the *E. coli* system and an MCS for integration of ARS, rDNA, selection marker and expression cassette modules. For this purpose *ARS* fragments are flanked by *Sac*II 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.

vector copy number. By this approach strains carrying up to 30 copies of an expression cassette have been isolated [66], similar to the high copy number observed in *H. polymorpha*.

3.1.1. Plasmids and available elements

Expression and integration vectors in *H. polymorpha* are composed of prokaryotic and yeast DNA [67]. 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 [68], an ARS sequence [69,70], the *URA3* gene [71], the *LEU2* gene [69], the *GAP* promoter region [72], or the rDNA cluster [7,20]. As stated before, the circular plasmids are not randomly integrated but recombine with genomic sequences represented on the vector.

For detailed information on the various *H. polymorpha* expression platforms, see [32,60,73]. Plasmids that have been successfully developed for industrial use of CBS4732-based strains include pFPMT121 (for production of phytase) and a derivative of pMPT121 (for production of the anti-coagulant hirudin) [60], the ranges of possible elements are included into the modular CoMed® vector system (see Fig. 4).

As multiple integration systems based on complementation of auxotrophic mutations, the plasmids AMpL1, AMIpLD1, and AMIpSU1 have been used in eliciting desired plasmid copy numbers in DL-1-derived recombinant strains. When an appropriate mutant strain is transformed with one of these plasmids under selective conditions, transformants with plasmid integrated in low (1–2), moderate (6–9), or high (up to 100) copy number can be rapidly selected [69]. Alternatively the G418 and hygromycin B resistance cassettes can be used as dominant selection markers allowing selection of transformants with copy numbers ranging from 1 to 50, in correlation to different drug concentrations [74,75].

Signal sequences may be fused to the target ORF (open reading frame) for protein secretion or for cell compartment targeting, such as the peroxisome, the vacuole, the endoplasmic reticulum, the mitochondria, or the cell surface. Available signal sequences include the peroxisomal targeting signals PTS1 and PTS2 [76], the repressible acid phosphatase (*PHO1*) secretion leader sequence [77], a *Schwanniomyces occidentalis*-derived

GAM1 [76,78], and the *S. cerevisiae*-derived *MFα1* sequence [5]. Glycosylphosphatidylinositol (GPI)-anchoring motifs derived from the GPI-anchored cell surface proteins, such as *HpSED1*, *HpGAS1*, *HpTIP1*, and *HpCWP1*, have been recently 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 at the cell surface [79]. One of the main advantages of heterologous gene expression in *H. polymorpha* is that this yeast has unusually strong promoters, the most widely employed of which are derived from genes of the methanol utilization pathway. These promoters include elements derived from the methanol oxidase (*MOX*), formate dehydrogenase (*FMD*), and dihydroxyacetone synthase (*DAS*) gene [5,76]. Other available but less frequently applied regulative promoters are derived from inducible genes encoding enzymes involved in nitrate assimilation (e.g., *YNT*1, *YNI*1, *YNR*1, which can be induced by nitrate and repressed by ammonium) [80], or the *PHO1* promoter of the gene encoding the enzyme acid phosphatase [77,81]. Examples of constitutive promoters are *ACT* [75], *GAP* [72], *PMA*1 [82], and *TPS*1 [83]. The *PMA*1 promoter even competes with the outstanding *AOX* promoter in terms of high expression levels; *PMA*1 is of interest in the co-expression of genes on industrial scale [8]. The performance of the *TPS*1 promoter is not linked to the use of a particular carbon source. In contrast to the constitutive promoters listed above, it can be applied at elevated temperatures, where its activity may be boosted even further [83]. The *H. polymorpha*-derived *FLD*1 gene encoding formaldehyde dehydrogenase has been characterized recently [81]. FLD1p is essential for the catabolism of methanol and shows 82% sequence identity with the Fld1p protein from *P. pastoris* and 76% identity with Fld1p from *C. boidinii*. The *FLD*1 promoter promises to be advantageous in that expression can be controlled at two levels: it is strongly induced under methylotrophic growth conditions, but shows moderate activity using primary amines as a nitrogen source. With these promising characteristics, the *FLD*1 promoter is expected to augment the existing range of *H. polymorpha* promoters [81]. The *GAP* promoter also showed a higher specific production rate and required a much simpler fermentation process than the *MOX* promoter-based HSA production system, implying that the *GAP* promoter can be a practical alternative to the *MOX* promoter in the large-scale production of some recombinant proteins [84].

For *P. pastoris* a similar set of elements is available. Most *P. pastoris* expression vectors harbour an expression cassette composed of a 0.9-kb *AOX1* promoter fragment, and a second short *AOX1*-derived transcription termination fragment [85,86], separated by a multiple-cloning site (MCS) for insertion of the foreign coding sequence. In addition, vectors are available where in-frame fusions of foreign proteins and a leader sequence for secretion can be constructed. Options are the *P. pastoris*-derived *PHO*1-pre-sequence or the *S. cerevisiae*-derived *MFα*1-prepro-sequence.

The strict dependence of the *AOX1* promoter on potentially hazardous methanol (see Section 1) has forced the use of alternative promoter elements that do not require methanol for activation. As such the *P. pastoris*-derived *GAP*, *FLD1*, *PEX8*, and *YPT1* promoters are available. The *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase (*GAP*) gene promoter provides strong constitutive expression on glucose at a level comparable to that seen with the *AOX1* promoter [87]. The *FLD1* gene encodes glutathione-dependent formaldehyde dehydrogenase, a key enzyme required for the metabolism of certain methylated amines as nitrogen sources and methanol as a carbon source [88]. The *FLD1* promoter can be induced by either methanol as a sole carbon source (and ammonium sulphate as a nitrogen source) or methylamine as a sole nitrogen source (and glucose as a carbon source). Thus, the *FLD1* promoter offers the flexibility to induce high levels of expression using either methanol or methylamine, an inexpensive non-toxic nitrogen source. Promoters of moderate strength are those derived from the *P. pastoris* *PEX8* and *YPT1* genes. The *PEX8* gene (formerly *PER3*) encodes a peroxisomal matrix protein that is essential for peroxisome biogenesis [89]. It is expressed at a low level on glucose and is induced modestly (3- to 5-fold) after shift of cells to methanol. The *YPT1* gene encodes a GTPase involved in secretion, and its promoter elicits a low constitutive level of expression on glucose, methanol, or mannitol as carbon source [90].

3.2. A. adeninivorans *and* Y. lipolytica

The first transformation system based on *A. adeninivorans* has been developed by Kunze et al. [91] and Kunze and Kunze [92] using the *LYS2* genes from *A. adeninivorans* and *S. cerevisiae* as selection markers. In these instances transformation vectors inconsistently either integrated into the chromosomal DNA or were of episomal fate displaying an altered restriction pattern. Therefore this system was replaced by an alternative one based on a stable integration of heterologous DNA into the ribosomal DNA (rDNA) [93]. For rDNA targeting it is equipped with an *A. adeninivorans*-derived 25S rDNA fragment. Further elements are selection markers like the *Escherichia coli*-derived *hph* gene inserted between the constitutive *A. adeninivorans*-derived *TEF*1 promoter and the *PHO5*-terminator conferring resistance to hygromycin B, or the *A. adeninivorans*-derived *ALEU2* and *AILV1* genes for complementation of a respective auxotrophic strain. The resulting transformants were observed to harbour 2–10 plasmid copies

stably integrated into the ribosomal DNA [93]. Transformants could be obtained of both the wild-type strain and mutant strains. For secretion a set of secretion leader sequences is available similar to those described before for the methylotrophs [94,95].

For industrial application of *Y. lipolytica* well-growing strains like W29 have been generated [10]. Descendents such as Po1d, Po1f and Po1h strains are genetically modified carrying non-reverting deletions of marker genes (*LEU2*, *URA3*) and of known extracellular proteases. The expression vectors used for transformation of *Y. lipolytica* are shuttle vectors like in the other yeast systems described before. Two major types of expression vectors are described for *Y. lipolytica*, differing by their mode of maintenance. Since episomal replicative vectors (presence of *Y. lipolytica*-derived ARS elements with co-localized centromeric and replicative functions) do not seem to meet stringent industrial requirements and demands [11,96]. The following description is restricted to the integrative vectors.

Integration of exogenous DNA into *Y. lipolytica* genome occurs mainly by homologous recombination, which is strongly stimulated by the linearization of the plasmid within the targeting region. In more than 80% of the cases, a single complete copy of the vector will be integrated at the selected site [10]. Integrated vectors exhibit a very high stability, as demonstrated by Hamsa and Chattoo [97]. The homologous integration of monocopy vectors into *Y. lipolytica* offers several advantages over the situation in other yeast expression systems, such as *P. pastoris*: (i) very high transformation efficiency, and (ii) a precise targeting of the monocopy integration into the genome. With these characteristics, the analysis of only a small number of transformants allows the selection of a correct integrant, and the performances of integrants from different experiments can be easily compared since their copy number and integration loci are identical. These characteristics facilitate further genetic engineering via directed mutagenesis, DNA shuffling or in vitro evolution for the improvement of constructs and of their products for industrial applications [98].

First attempts to increase the copy number in *Y. lipolytica* used homologous multiple integrations into the ribosomal DNA (rDNA) cluster, together with a defective selection marker, i.e., the *ura3d4* allele [99,100]. Using this combination of transformants carrying up to 60 integrated copies could be obtained, present as tandem repeats at one or two genomic sites. However, only around ten copies were found to be stable in cases of detrimental gene products. Other potential genomic regions for targeting are the Ylt1 retrotransposon (up to 35 copies) [101] with very large (714 bp long) terminal repeats (LTRs) and the ''zeta'' sequences, which exist also as solo copies (up to 60) [102].

The native form of the *XPR2* promoter (p*XPR2*) was initially applied to the expression of heterologous proteins [97,103–106]. However, a complex regulation imposed restriction on general industrial use: it is active at pH above 6, and its full induction requires high levels of peptones in the culture medium [107]. The functional dissection of the p*XPR2* showed that one of its upstream activating sequences, UAS1, was poorly affected by environmental conditions [108,109]. Consequently, this element was used to construct a hybrid promoter, composed of four tandem copies of the UAS1, inserted upstream from a minimal *LEU2* promoter (reduced to its TATA box). This recombinant promoter, *hp4d*, is almost independent from environmental conditions such as pH, carbon and nitrogen sources, and presence of peptones [110,111]. *Hp4d* is able to drive a strong expression in virtually any medium. It retains yet unidentified elements that drive a growth phase-dependent gene expression, since *hp4d*-driven heterologous gene expression was found to occur at the beginning of stationary phase [111,112]. This newly acquired characteristic enables a dissociation of growth and expression phases. *Hp4d* has been used successfully for the production of various heterologous proteins in *Y. lipolytica* [111–116].

Two strong constitutive promoters, derived from the *Y. lipolytica TEF* and *RPS7* genes have been isolated and described by Müller et al. [106]. These promoters are particularly suited for the isolation of new enzyme genes by expression cloning, but not recommended for heterologous production per se.

A number of inducible promoters with interesting properties has been described. Dominguez et al. [117] reported the use of the bidirectional metallothionein promoter, but the requirement of metal salts for induction imposes restriction on general use. The capacity of *Y. lipolytica* to grow on hydrophobic substrates promoted the search for promoters of genes for key enzymes from this pathway. As such, promoters from isocitrate lyase (*ICL1*), 3-oxo-acyl-CoA thiolase (*POT1*), and acyl-CoA oxidases (*POX1*, *POX2* and *POX5*) were isolated and tested. They have been compared to the native promoters of glycerol-3-phosphate dehydrogenase and alkaline protease, and to the recombinant promoter *hp4d*, in terms of activity and regulation by various carbon sources [118]. p*ICL1*, p*POT1* and p*POX2* were found to be the strongest inducible *Y. lipolytica* promoters. They are highly inducible by fatty acids and alkanes, and repressed by glucose and glycerol. p*ICL1* is also inducible by ethanol and acetate, but it is not completely repressed in the presence of glucose and glycerol. p*ICL1* and p*POX2* have been used successfully for heterologous production [102,112,119–121]. However, general industrial use of these new inducible promoters still faces some problems: on one hand p*ICL1* exhibits the mentioned high basal expression level of in the presence of certain carbon sources, on the other hand activation of p*POT1* and p*POX2* relies on hydrophobic inducers. Their presence in a

cultivation broth may be incompatible with efficient protein production or purification.

3.3. Wide-range integrative yeast expression vector system

Since obviously no single yeast-based expression platform exists which is optimal for every protein it is useful to assess several platform candidates in parallel for optimal product characteristics. The availability of a vector system that could be targeted to the various candidates would greatly facilitate a comparative assessment. A suited vector must contain a targeting sequence, a promoter element and a selection marker that function in all selected organisms. These criteria are fulfilled by the wide-range integrative yeast expression vector systems based on *A. adeninivorans*- and *H. polymorpha*-derived elements [7,20–22]. The basic vectors harbour the conserved NTS2-ETS-18SrDNA-ITS1 region from *H. polymorpha* or the 25S rDNA region from *A. adeninivorans* for targeting, the *A. adeninivorans*-derived *TEF1* promoter for expression control of the reporter sequence, and the *E. coli*-derived *hph* gene conferring resistance against hygromycin B for selection of recombinants. Heterologous gene expression was assessed using the green fluorescent protein (GFP)-, the *Aspergillus*-phytase- or the *E. coli lacZ*- reporter gene. The plasmids were found to be integrated into the genome of *A. adeninivorans*, *S. cerevisiae*, *H. polymorpha*, *P. pastoris*, *P. stipitis*, *Debaryomyces hansenii* and *D. polymorphus*. All recombinant strains exhibited heterologous gene expression.

Since vector systems of different yeast species are based on different basic vectors it is very difficult to exchange single cassettes between the yeast systems. To eliminate this disadvantage the CoMed® vector system was established, containing the pCoMed® basic vector for integration of ARS, selection markers, rDNA sequences and expression cassettes. For this purpose the single modules are flanked by the same restriction sites and are integrated in the same location of the basis vector (Fig. 4). In this system various modules can be integrated. If for instance the combination of rDNA and the *ALEU2* gene is combined, a range of yeasts with this auxotrophy can be targeted. The expression cassette is inserted as a final construct. A range of such cassette elements exists harbouring a promoter of choice (among others the *TEF1* promoter mentioned before), and a *PHO5* terminator separated by a multiple-cloning site. The general design of the plasmid CoMed® is provided in Fig. 4, a selection of components is given in Table 1.

4. Product and process examples

Due to the divergent track record and tradition of the four selected organisms it is almost impossible to compare their capabilities and specific advantages by taking established processes into consideration. Several heterologous genes were assessed for expression in *A. adeninivorans*. As a first example the *XylE* gene from *Pseudomonas putida* encoding the catechol-2,3-dioxygenase was fused to the *AILV1* promoter for expression analysis [91,92]. Further successful examples include the expression of *GFP* and *HSA* gene sequences inserted into the basal vectors pAL-HPH1 and pAL-ALEU2m containing the hygromycin B expression cassette or the *ALEU2* gene as selection marker. The resulting expression vectors contain the coding sequences inserted between the strong constitutive *TEF1* promoter and the *PHO5* terminator. 1–2 linearized plasmid copies integrated specifically into the chromosomal 25S rDNA region. In case of *GFP* expression the recombinant protein was localized in the cytosol, rendering the cells fluorescent. In case of *HSA*, expression is based on an ORF including the native signal sequence at the 5′-end. Accordingly, the recombinant HSA was secreted to more than 95% into the culture medium. In initial fermentation trials of a single-copy transformant on a 200 ml shake-flask scale, maximal HSA product levels of 50 mg l^{-1} were observed after 96 h of cultivation. Budding cells as well as mycelia secreted similar levels, demonstrating a morphology-independent productivity [95,122].

In addition to the *TEF1* promoter the strong constitutive *AHSB4* promoter was successfully tested for its suitability for the heterologous gene, resulting in similar expression levels. In order to facilitate the integration into the expression plasmids a modified *AHSB4* promoter sequence (*AHSB4m* promoter) was applied [122].

For construction of a recombinant biocatalyst, *A. adeninivorans* was equipped with the genes *phb*A, *phb*B and *phb*C of the polyhydroxyalkanoate (PHA) biosynthetic pathway of *Ralstonia eutropha*, encoding β-ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHA synthase, respectively. *A. adeninivorans* strains initially transformed with the PHA synthase gene (*phb*C) plasmids alone were able to produce PHA. However, the maximal content of the polymer detected in these strains was just 0.003% (w/w) poly-3-hydroxybutyrate (PHB) and 0.112% (w/w) poly-3-hydroxyvalerate (PHV). The expression of all three genes (*phb*A, *phb*B, *phb*C) resulted in small increases in the PHA content only. However, under controlled conditions, using minimal medium and ethanol as the carbon source for cultivation, the recombinant yeast was able to accumulate up to 2.2% (w/w) PHV and 0.019% (w/w) PHB ([9,123] and Fig. 5).

To date more than 40 heterologous proteins have been produced in *Y. lipolytica*. The range of proteins includes several examples of human origin, among others blood coagulation factor XIIIa, insulinotropin, epidermal growth factor (EGF) and single-chain antibodies

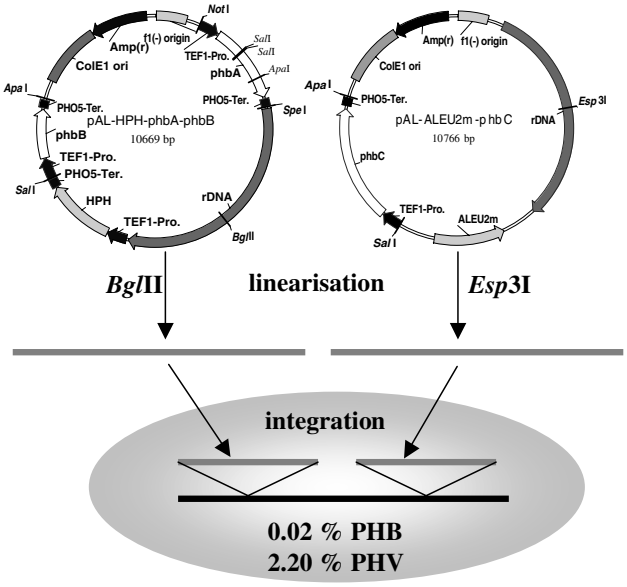

Fig. 5. Transformation procedure based on simultaneous integration of the plasmids pAL-HPH-phbA-phbB and pAL-ALEU2m-phbC into the 25S rDNA of *A. adeninivorans* G1211 (*aleu2*). The two plasmids pAL-HPH-phbA-phbB and pAL-ALEU2m-phbC containing the expression cassettes with *phbA, phbB* and *phbC* genes are linearised by *Bgl*II or *Esp*3I digestion, respectively. The resulting fragments flanked by 25S rDNA sequences are co-integrated into the 25S rDNA by homologous recombination. Transformants are selected either by resistance to hygromycin B (pAL-HPH-phbA-phbB [93]) or the complementation of the *aleu2* mutation (plasmid pAL-ALEU2m-phbC [95]).

(scFvs). For a comprehensive list the reader is referred to [11]. Product yields range from 100 mg l^{-1} of *Trichoderma reesei*-derived endoglucanase I when using the *XPR2* promoter for expression control [124], to 160 mg l^{-1} for bovine prochymosin and to 1 g l^{-1} using *hp4*d promoter for expression control [111].

The following is a short summary of *H. polymorpha*-based processes. Launched products include IFNα-2a [125], insulin (Wosulin by Wockhardt), several hepatitis B vaccines, and a bakery ingredient (hexose oxidase contained in Grindamyl-Surebake by Danisco) [126]. A few industrially relevant examples will be briefly summarized. For a more detailed description of fermentation and purification procedures the reader is referred to [127] and [128].

Once stable recombinant integrants have been generated production strain candidates are identified from a background of non-producers or strains of low or impaired productivity. The subsequent 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 h. Due to the inherent versatile characteristics of the two methanol-inducible promoters fermentation modes vary, for the most part in the supplemented carbon source: glycerol, methanol, glucose, and combinations thereof may be selected. The ability to achieve high yields of a recombinant product, expressed from a methanol pathway promoter without the addition of methanol, is a unique feature of the *H. polymorpha* system (see Section 1) [5,60]. In contrast, activation of these promoters in the related yeast *P. pastoris* is strictly dependent on the presence of methanol [12].

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 *MF*α1 prepro-sequence/hirudin fusion gene under control of the *MOX* promoter [78,129,130]. 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 was started with 3% (w/v) glycerol at the beginning of fermentation. After consumption of the carbon source after 25 h the pO_2-controlled feeding mode was initiated, resulting in a glycerol concentration between 0.05 and 0.3% (w/v) (derepression of the *MOX* promoter). The fermentation run was terminated after 36 h of derepression (total fermentation time of 72 h). Then the broth was harvested and the secreted product 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 [131]. The producer strain harboured 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 was fermented on a 50-l scale. The product-containing cells were generated via a two fermentor cascade, consisting of a 5-l seed inoculating the 50-l main fermentor. The initial steps of fermentation closely followed those described for the production of hirudin. At the beginning cultivation was performed with a glycerol feeding 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 derepression phase was then followed by a batchwise feeding with methanol in the final fermentation mode. The product increased to amounts in the multigram range. It consists of a lipoprotein particle in which the recombinant HBsAg is inserted into host-derived membranes. As pointed out in the introduction, 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 [131–133]. 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 detailed in [131–133].

For the production of phytase, *H. polymorpha* has been used in a particularly efficient process [16,134], a prerequisite for an economically competitive production of a technical enzyme. In this development all steps and components of strain generation, fermentation, and purification are dictated by a rationale of efficiency and cost-effectiveness. This also applies to the definition of the fermentation process using glucose as the main carbon source.

A strain was generated in which the phytase sequence is under control of the *FMD* promoter. Subsequent supertransformation yielded strains with up to 120 copies of the heterologous DNA, enabling a gene dosage-dependent high productivity. Then a fermentation procedure was developed to achieve high levels of enzyme production. Significantly, it was found that the use of glycerol as the main carbon source was not required in this case, but that could be substituted by low-cost glucose. The active status of the *FMD*-promoter was maintained by glucose starvation (fermentation with minimal levels of continuously fed glucose). At a 2000-l scale, fermentation with glucose as the sole carbon source led to high product yields and an 80% reduction in raw material costs compared to glycerol-based fermentations [16,134]. Strains were found to produce the recombinant phytase at levels ranging up to 13.5 g l^{-1} [16,134]. 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%.

P. pastoris has become a very popular academic tool for the production of recombinant proteins; several hundred examples exist in the literature. In contrast, the range of launched products manufactured in this organism is still very limited. The proteins produced include insulin [135], lipases [136] and HSA, for which a process on a 10,000-l scale has been designed. In most of the examples the *AOX1* promoter was applied to the control of heterologous gene expression and a general outline is given in the following.

With its preference for respiratory growth, *P. pastoris* can be cultured at extremely high densities (150 g l^{-1} dry cell weight; 500 OD_{600} U ml^{-1}) in the controlled environment of the fermentor, comparable to *H. polymorpha*. High-cell density growth is especially important for secreted proteins, as the concentration of product in the medium is roughly proportional to the concentration of cells in the culture. The level of transcription initiated from the *AOX1* promoter can be 3–5 times greater in cells fed methanol at growth-limiting rates compared to that in cells grown in excess methanol. The *P. pastoris* expression strains can easily be scaled-up from shake-flask to high-density fermentor cultures. Considerable effort has gone into the optimization of heterologous protein expression techniques, and detailed fed-batch and continuous-culture protocols are available [13,65,137]. In general, fermentation of P_{AOX1}-controlled expression strains follows a design similar to that described for the ''two carbon source fermentation'' of HBsAg-producing *H. polymorpha* strains. They are grown initially in a defined medium containing glycerol as its carbon source. During this time, biomass accumulates, but heterologous gene expression is fully repressed (in contrast to the situation in *H. polymorpha*). Upon depletion of glycerol, a transition phase is initiated in which additional glycerol is fed to the culture at a growth-limiting rate. Finally, methanol or a mixture of glycerol and methanol is fed to the culture to induce expression. The growth medium for *P. pastoris* in recombinant protein production fermentations is ideal for large-scale production because it is inexpensive and defined, consisting of pure carbon sources (glycerol and methanol), biotin, salts, trace elements, and water, again comparable to the media described for *H. polymorpha*. This medium is free of undefined ingredients that can be sources of pyrogens or toxins and is, therefore, compatible with the production of human pharmaceuticals. Also, since *P. pastoris* is cultured in media with a relatively low pH and methanol, it is less likely to become contaminated by most other microorganisms.

A detailed outline of product recovery and downstream processing has been provided elsewhere for secreted and intracellular products from both expression systems. An individual procedure has to be defined for every process to be developed. Especially in the case of secreted compounds, fermentation and primary product recovery are intimately linked. This interface of upstream and downstream processing is in some instances the objective of a successful integrated bioprocess development [138,139], among other examples for production of aprotinin variants in *H. polymorpha* [140] and for production of HSA in *P. pastoris* [15].

5. Comparative aspects

A reliable comparison of yeast-based expression platforms is difficult. Only a few studies exist in the literature that describes the comparative expression of defined identical reporter genes in several yeasts. One of them was the comparative production of six selected fungal enzymes in *S. cerevisiae*, *H. polymorpha*, *Kluyveromyces*

lactis, *Schizosaccharomyces pombe* and *Y. lipolytica* [106]. In another study production of human IL-6 was compared in *A. adeninivorans, H. polymorpha* and *S. cerevisiae*. All three organisms produced the recombinant protein in high amounts. The MFα1-IL6 precursor was found to be correctly processed in *A. adeninivorans* but not in *H. polymorpha* and *S. cerevisiae* (E. Böer et al., unpublished observations). Nevertheless the conclusiveness of such comparison is limited.

Protein glycosylation represents an important type of modification. Detailed analyses of glycosylation have been performed for *S. cerevisiae, H. polymorpha* and *P. pastoris*. Like all yeasts they are capable of *N*- and *O*-glycosylation, but subtle differences exist. *N*-glycosylation is restricted to the "high-mannose type". In *S. cerevisiae* a tendency of "hyperglycosylation" is observed – a heterogeneous addition of 50–150 mannose residues to the glycosylation core. In the two methylotrophs this is much less pronounced but is also observed in several cases. On average the outer mannose chain length ranges between 8 and 14 [6,7]. In *Y. lipolytica* a single example exists with an observed chain length of 8-10 mannose [11]. In case of *S. cerevisiae* the terminal mannose residue is linked by an α1,3 bond which is considered to be allergenic. Instead, a non-allergenic α1,2 bond is present in the methylotrophs. In *A. adeninivorans O*-glycosylation was found in case of AFet3p to be restricted to the budding yeast status [141]. For both methylotrophs modified hosts that add a "humanized" pattern of *N*-glycosylation to recombinant proteins have recently been developed (H.A. Kang, pers. commun.). All yeasts selected for the present comparison are representatives of "non-conventional yeasts", a collective but misleading term for non-*Saccharomyces* species. All of them are distinguished by growth on a broad range of carbon sources. Accordingly they have developed special metabolic pathways. Genes of these pathways are potential sources for promoter elements with superior characteristics.

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