Chapter 27
Arxula adeninivorans (Blastobotrys adeninivorans) – A Dimorphic Yeast of Great Biotechnological Potential

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Content

Abstract The dimorphic ascomycetous yeast *Arxula adeninivorans* exhibits some unusual properties. Being a thermo- and halotolerant species it is able to assimilate and ferment many compounds as sole carbon and/or nitrogen source. It utilises n-alkanes and is capable of degrading starch. Due to these unusual biochemical properties *A. adeninivorans* can be exploited as a gene donor for the production of enzymes with attractive biotechnological characteristics. Examples of *A. adeninivorans*-derived genes that are overexpressed include the *ALIP1* gene encoding a secretory lipase, the *AINV* encoding invertase, the *AXDH* encoding xylitol dehydrogenase and the *APHY* encoding a secretory phosphatase with phytase activity.

The thermo- and halotolerance as well as differential morphology-dependent glycosylation and the secretion characteristics render *A. adeninivorans* attractive as host for heterologous gene expression. A transformation system has been established based on homologous integration of linearised DNA fragments. Successful expression examples like that of the *E. coli*-derived *lacZ* gene, *GFP* and human *HSA* and *IL6* genes add to the attraction of *A. adeninivorans* as host for heterologous gene expression.

Keywords Dimorphic yeast, *Arxula adeninivorans*, phosphatase, heterologous gene expression, thermotolerance, halotolerance

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27.1 Introduction

Yeasts are simply organized ubiquitous unicellular eukaryotes that are able to adapt rapidly to alterations of environmental conditions. In addition to the traditional baker's yeast *Saccharomyces cerevisiae* a wide range of non-conventional yeast species exists with attractive characteristics and growth properties. These species can be exploited for biotechnological applications and can serve as suitable model organisms for plant or animal research. They are either used as donor for genes encoding interesting gene products or employed as excellent hosts for the production of recombinant proteins. The range of yeast species that have been developed as platforms for heterologous gene expression includes the traditional baker's yeast *Saccharomyces cerevisiae*, *Kluyveromyces lactis, Pichia pastoris, Yarrowia lypolytica, Hansenula polymorpha*, *Arxula adeninivorans* (Gellissen, 2005; Gellissen et al., 2005; Wolf, 1996; Wolf et al., 2003) and others described in this book.

The first description of *A. adeninivorans* was provided by Middelhoven et al. (1984) who selected a yeast species from soil by enrichment culturing designating it as *Trichosporon adeninovorans*. A particular strain CBS 8244T was found to exhibit unusual biochemical activities being able to assimilate a range of amines, adenine and several other purine compounds as sole energy and carbon source.

At the same time a second strain, LS3 (PAR-4) with characteristics similar to the CBS strain (Gienow et al., 1990) was isolated from wood hydrolysates in Siberia (Kapultsevich, Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia).

In 1990, three additional *Tr. adeninovorans* strains were isolated from chopped maize herbage ensiled at 25 or 30°C in the Netherlands, yet another four strains were detected in humus-rich soil in South Africa (Van der Walt et al., 1990). A new genus name *Arxula* Van der Walt, M.T. Smith & Yamada (*Candidaceae*) was proposed for all these strains. They all share properties like nitrate assimilation and xerotolerance. All representatives of the newly proposed genus are ascomycetous, anamorphic and arthroconidial (Van der Walt et al., 1990).

Kurtzmann and Robnett (2007) accomplished phylogenetic analyses of the ascosporic yeast genera *Sporopachydermia, Stephanoascus, Trichomonascus, Wickerhamiella* and *Zygoascus* and the associated anamorphic genera *Arxula, Blastobotrys, Sympodiomyces* and *Trigonopsis* comparing sequences derived from the large-subunit rDNA genes, the mitochondrial small-subunit rDNA genes, and the genes for cytochrome oxidase II. They deduced that *Arxula, Blastobotrys* and *Sympodiomyces* are members of the *Trichomonascus* clade, with the genus *Blastobotrys* having taxonomic priority for anamorphic states (Fig. 27.1).

The genus *Blastobotrys* includes now the both type species of the genus *B. terrestris* (Van der Walt & Johanssen) Kurtzman & Robnett comb. nov. (Basionym: *Arxula terrestris*) and *B. adeninivorans* (Middelhoven, Hoogkamer Te-Niet & Kreger van Rij) Kurtzman & Robnett comb. nov. (Basionym: *Arxula adeninivorans*).

Eukaryot **(Superkingdo)**
Fungi **(Kingdom**
Ascomycot **(Phylu)**
Saccharomycotin **(Subphylu)**
Saccharomycet **(Clas)**
Saccharomycetal **(Order)**
Mitospori *Saccharomycetal*
Blastobotry **(Genus**
Blastobotry adeninivoran **(Specie)**

Fig. 27.1 Taxonomy of *B. adeninivorans* (synonym: *A. adeninivorans*) (Kurtzmann and Robnett, 2007)

27.2 Physiology and Temperature Dependent Dimorphism

A detailed physiological description of the yeast was provided by Gienow et al. (1990), Middelhoven (1993) and Middelhoven et al. (1984, 1991, 1992). *A. adeninivorans* is able to assimilate nitrate like *H. polymorpha* employing nitrate reductase and nitrite reductase for metabolism. It can utilize a range of compounds as sole energy and carbon source including adenine, uric acid, butylamine, pentylamine, putrescine, soluble starch, melibiose, melicitose, propylamine or hexylamine. It rapidly assimilates all sugars, polyalcohols and organic acids used in conventional carbon compound assimilation tests, except for L-rhamnose, inulin, lactose, lactate and methanol. Likewise all 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 energy, carbon and nitrogen source, furthermore many primary n-alkylamines and terminal diamines. In case of alcohols, dialcohols, carboxylic acids, dicarboxylic acids and other nitrogen-free analogous compounds, metabolic intermediates are assimilated. In addition, *A. adeninivorans* degrades some phenols, hydroxybenzoates, tannic acid and is able to assimilate urotropine as sole nitrogen source (Middelhoven and van Doesburg, 2007).

For substrate utilization *A. adeninivorans* produces numerous secretory enzymes including RNases, proteases, glucoamylase, lipase, tannase, some acid phosphatases, trehalase, some cellobiases, invertase, -glucosidase, xylosidase and phytase. Table 27.1 lists these secretory enzymes and summarizes some of their properties.

Special features of biotechnological impact are the thermotolerance- and temperature-dependent dimorphism which is especially pronounced in the Siberian wild strain *A. adeninivorans* LS3. This strain can grow at temperatures of up to 48°C without previous adaptation to elevated temperatures and is able to survive some hours at a temperature of 55°C (Böttcher et al., 1988; Wartmann et al., 1995a). Strain LS3

Table 27.1 Properties of secretory enzymes of *A. adeninivorans* (Böer et al., 2004b, 2005b; Büttner et al., 1987, 1988, 1989, 1990a, c, 1991a, b; Büttner and Bode, 1992; Büttner et al., 1992a, b; Kunze and Kunze, 1994b; Sano et al., 1999; Wartmann et al., 1995b)

	Optimum		Molecular mass	
Enzyme	Temperature	pH	k_m value	(Da)
Glucoamylase (1.4-α-D-glucan glucohydrolase, EC 3.2.1.3)	60–70°C	4.0–5.0	1.2 g/L for starch 11.1 mM for maltose	225,000
Acid phosphatase I and II (ortho-phosphoric-monoester phospho-hydrolase, EC 3.1.3.2)				
I	50–55°C	5.2–5.5	3.5 mM for p-nitro-phenylphosphate	320,000
II	50–55°C	5.2–5.5	5 mM for p-nitro-phenylphosphate	250,000
Trehalase (α,α-trehalose-gluco-hydrolase, EC 3.2.1.28)	45–55°C	4.5–4.9	0.8–1.0 mM for trehalose	250,000
Cellobiase I and II (β-D-gluco-sidase, EC 3.2.1.21)				
I	60–63°C	4.5	4.1 mM for cellobiose	570,000
II	60–63°C	4.5	3.0 mM for cellobiose	525,000
Invertase (β-D-fructofura-noside fructohydrolase, EC 3.2.1.26) 36 mM raffinose	50–60°C	4.5	40–60 mM for sucrose	600,000
β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37)	60°C	5.0	0.23–0.33 mM for p-nitro-phenyl-β-xylopyranoside	60,000
3-Phytase (*myo*-inositol hexakis phosphate 3-phosphohydro-lase EC 3.1.3.8)	75°C	4.5	0.23 mM for phytata	n.d.
Lipase (triacylglycerol acylhydro-lases, EC 3.1.1.3)	30°C	7.5	0.4 mM *p*NP-caprate	100,000
Tannase (tannin acyl hydrolase, EC 3.1.1.20)	35–45°C	5.0–6.5	0.14 mM for gallotannin	320,000

exhibits a temperature-dependent morphological dimorphism. At temperatures above 42°C a reversible transition from budding cells to mycelial forms is induced. Budding is re-established when cultivation temperature is decreased below 42°C (Fig. 27.2).

Wartmann et al. (2000) selected mutants with altered dimorphism characteristics. These mutants grow already as mycelia at 30°C thus enabling a distinction between temperature-mediated and morphology-related effects on gene expression and protein accumulation. In analogy to other dimorphic yeasts *A. adeninivorans* budding cells and mycelia differ in their contents of RNA and soluble protein and in their dry weight. During the middle and the final phases of the exponential growth mycelia are found to be of lower RNA and protein content. In contrast, synthesis of secreted proteins including the enzymes glucoamylase and invertase is more pronounced in mycelia resulting in a two-fold higher extracellular protein accumulation. This indicates that morphology, rather than temperature, is the decisive factor for the observed differences (Table 27.2).

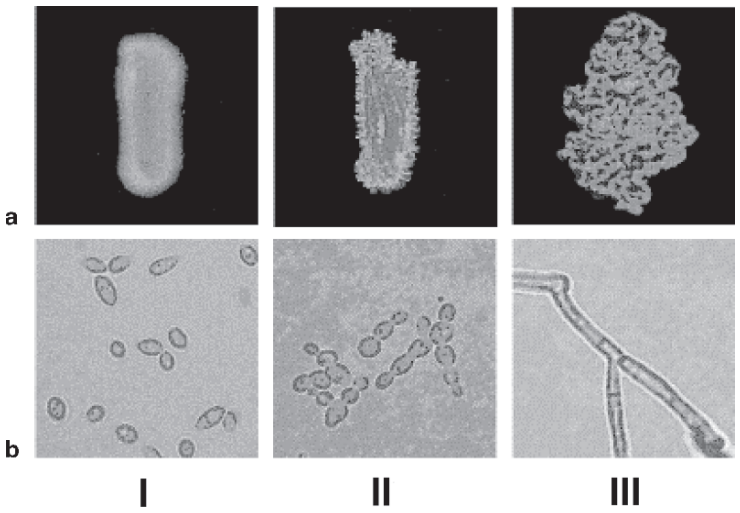


Fig. 27.2 (**a**) Colony form and (**b**) cell morphology of *A. adeninivorans* LS3 grown at 30°C (I), 42°C (II) and 45°C (III). The cells were cultured in YEPD medium for 18 h

Table 27.2 DNA and RNA content, dry weight and amount of soluble protein of *A. adeninivorans* LS3 cultured at 30°C (budding cell) and at 45°C (mycelium) and of *A. adeninivorans* 135 cultured at 30°C (mycelium) in yeast minimal medium (Tanaka et al., 1967) with 1% maltose as carbon source (Wartmann et al., 2000). The values are means ± SD from three separate cultures each with three batches in parallel

Budding cell	Mycelium LS3-30°C	Mycelium LS3-45°C	135-30°C
Content (fg)			
DNA	25.3 ± 0.5	23.6 ± 2.4	24.8 ± 0.9
RNA (45 h)	118.0 ± 15.2	56.4 ± 9.1	44.7 ± 8.8
max. RNA	142.5 ± 18.0 (45 h)	73.0 ± 21.0 (36 h)	57.5 ± 9.6 (36 h)
Dry weight (pg, 45 h)	18.2 ± 0.2	22.4 ± 0.8	23.3 ± 0.3
Soluble protein (fg, 45 h)	169.2 ± 16.3	107.5 ± 20.2	76.9 ± 0.3
Max. soluble protein (fg)	234 ± 5.5 (60 h)	150.1 ± 17.9 (60 h)	125.8 ± 11.6(60 h)*A.*

A. adeninivorans is provided with two transport systems that differ in iron affinity. A strong correlation exists between the morphological status and the iron uptake. In the presence of high Fe(II) concentrations (>2 μM), budding cells accumulate iron concentrations up to seven-fold higher than those observed in mycelia, while at low Fe(II) concentrations (< 2 μM), both cell types accumulate similar amounts of iron. The copper-dependent Fe(II) oxidase (Afet3p) and the respective *AFET3* gene, components of the high affinity transport system, were analyzed in more detail. In this case gene expression strongly depends on iron concentration but is independent

of the morphological stage. However cell morphology was found to influence the posttranslational modifications of Afet3p, 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 (Wartmann et al., 2002b).

In addition to temperature the presence of compounds like Cd^{2+}, tocopherol, NaCl and tunicamycin as well as anaerobic cultivation conditions cause alterations in the cell morphology whereas dimorphism is not affected by Ca^{2+}, pH-value, carbon source or substrate limitations (Table 27.3).

A further interesting property of *A. adeninivorans* is its osmotolerance. It can grow in minimal as well as rich media containing up to 3.32 osmomol kg^{-1} H_2O in presence of ionic (NaCl), osmotic (PEG400) and water stress (ethylene glycol). In strain LS3 the influence of NaCl on the growth characteristics was investigated in more detail. Supplementation with up to 3.4 M (10%) NaCl was of limited influence on growth only. However, at NaCl concentrations higher than 3.4 M a decrease of the specific growth rate, a longer adaptation phase and a lower cell count during the stationary growth phase was observed (Yang et al., 2000).

As in other yeast species, this tolerance is elicited by components of the high osmolarity glycerol (HOG) response pathway. However, in contrast to organisms of moderate osmo-resistance, which activate the HOG pathway by enzyme phosphorylation only, this species of high osmo-resistance also induces the expression of HOG pathway genes, such as MAPKK kinase-encoding *ASTE11* and MAP kinase-encoding *AHOG1*. Phosphorylated Ahog1p induces the expression of genes for the synthesis of compatible solutes, such as glycerol, erythritol and

Table 27.3 Environmental factors and their influence on the dimorphism of *A. adeninivorans* LS3

Transition budding cells → mycelia	⇒ Temperature ≥ 43°C
	⇒ 0.1 mM $CdSO_4$
	⇒ Tocopherol
Transition mycelia → budding cells ⇒ temperature < 43°C	
	⇒ NaCl Concentration ≥ 10%
	⇒ Anaerobic conditions
	⇒ Tunicamycin ≥ 8 μg mL^{-1}
Without influence	⇒ pH value
	⇒ Cultivation media (YEPD, YMM)
	⇒ Carbon source (glucose, fructose, maltose, sucrose, xylose, cellobiose, glycerol)
	⇒ Sub- and emers cultivation
	⇒ Substrate limitation
	⇒ Ca^{2+}

mannitol. While glycerol and erythritol levels correlate directly with the osmolarity of the culture media, intracellular mannitol is accumulated 50 fold in an osmolarity-independent manner. The combination of these effects seems to provide a better adaptation during the transition from low to high osmolarity conditions (Böer et al., 2004a).

27.3 Genetics and Molecular Biology

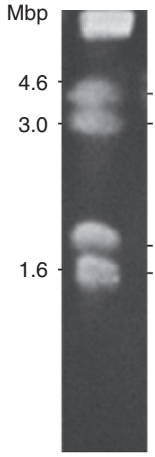
The DNA content of *A. adeninivorans* cells is similar to that of haploid cells of *S. cerevisiae* and other ascomycetous yeasts (Gienow et al., 1990; Samsonova et al., 1996; Wartmann et al., 2000). A relatively high frequency of auxotrophic mutants that is obtained after nitrosoguanidine mutagenesis (Samsonova et al., 1989, 1996), quantitative analysis of chromosomal DNA and determination of genome size (Gienow et al., 1990) further sustain the conclusion for *A. adeninivorans* to be a haploid organism.

The complexity of the nuclear genome of *A. adeninivorans* was analyzed by DNA re-association studies; furthermore karyotyping was performed by pulsed field gel electrophoresis (PFGE) by Gienow et al. (1990) and Kunze and Kunze (1994a). Genome sizes of 16.1×10^9 and 16.9×10^9 Da were calculated from reassociation kinetics of chromosomal DNA from both, *A. adeninivorans* wild type strains LS3 and 8244T, respectively. These are the highest values reported for a yeast species so far - much higher than the 9.2×10^9 Da determined for *S. cerevisiae* under identical conditions. The share of repetitive sequences was determined as 33.1% in LS3 and 35.9% in CBS 8244T. These values are higher than those reported for other yeasts, too. Karyotype polymorphisms were observed among the wild type strains tested demonstrating the existence of four chromosomes ranging between 1.6 and 4.6 Mb in size.

Genetic studies were promoted by the isolation of mutants and by the development of special techniques. After UV-induced mutagenesis or after treatment with nitrosoguanidine a large number of auxotrophic mutants and of mutants with an altered catabolite repression (resistance to 2-deoxy-D-glucose) have been selected and characterized (Böttcher and Samsonova, 1983; Büttner et al., 1990b; Samsonova et al., 1989, 1996).

Since no sexual cycle could be observed, mapping techniques based on parasexual mechanisms had to be applied to establish a genetic map of *A. adeninivorans*. After polyethylene glycol-induced fusion of spheroplasts heterozygous diploids were obtained from auxotrophic mutants of strains LS3 and CBS8244T (Büttner et al., 1990b; Samsonova et al., 1996). Segregation of these diploids was achieved by treatment with benomyl, a drug known to induce haploidization, without affecting other mitotic recombination events (Böttcher and Samsonova, 1983). This permitted the linkage analysis of various markers.

In this way 32 genes could be assigned to four linkage groups thus meeting the chromosome number of the *A. adeninivorans* genome. This was confirmed by



Chromosomes	1	2	3	4
	ARG7	*MET2*	*ARG4*	*ARG6*
	CYS1	*MET6*	*ADE1*	*ARG1*
	LEU2	*MET17*	*INO1*	***AILV1***
	LEU4	*ade-96*	*AINV*	*AEFG1*
	LYS5	*ade-109*		*AFET3*
	LYS2	*NIC4*		*APHO1*
	LYSI 4	***GAA***		
	PRO1	*TEF1*		
	PRO3	*ATRP1*		
	ADE2			
	NIC1			
	NIC2			
	NIC3			
	NIC8			
	PDX			
	ARFC3			
	AHSB4			
	AHOG1			
	ALIP1			
	AXDH			

Data obtained by linkage group analyses are normal bold, by PFGE are underlined and by linkage group analyses as well as PFGE are extra bold

Fig. 27.3 *A. adeninivorans* chromosomes separated by PFGE and chromosomal gene localization by linkage group analyses and PFGE followed by DNA hybridization

relating the analyzed 32 auxotrophic mutations to particular chromosomes by PFGE and subsequent DNA hybridization with specific probes (Samsonova et al., 1996; Fig. 27.3). Sequencing of the genome of strain LS3 is approaching completion at the time this manuscript was written (Gaillardin, personal communication).

27.4 *Arxula adeninivorans* as Gene Donor

Several *A. adeninivorans* genes were isolated from gene libraries containing either cDNA or chromosomal DNA from *A. adeninivorans* strain LS3 by PCR amplification with specific consensus primer sequences, among others *AEFG1, AFET3, AHOG, AHSB4, AINV, ALIP1, ALYS2, APHO1, ARFC3, ATAL, AXDH* and *TEF1*as well as the complete rDNA repeat (Böer et al., 2004a, 2004b, 2005b, 2005c; El Fiki et al., 2007; Kaur et al., 2007; Kunze and Kunze, 1996; Rösel and Kunze, 1995, 1996; Steinborn et al., 2005; Stoltenburg et al., 1999; Wartmann et al., 2001, 2002b, 2003a).

The presence of introns is more common in the *A. adeninivorans* genome than in other yeasts like *S. cerevisiae*. *AHSB4, ARFC1* and *AHOG1* were found to contain an intron. The comparison of 5′-splice site (DS/GUARGU), branch site (HRCUAAC) and 3′-splice site (HAG/R) sequences demonstrate that the resulting consensus sequences are similar to that of *S. cerevisiae* and filamentous fungi (Böer et al., 2005a).

The complementation of respective *E. coli* and *S. cerevisiae* mutants was used as an approach for the isolation of additional genes, namely the *ALYS2*, *AILV1, ALEU2* and *ATRP1* genes which are suitable selection markers for the *A. adeninivorans*-based platform (Kunze and Kunze, 1996; Steinborn et al., 2007b; Wartmann et al., 1998, 2003b).

The *GAA* gene encoding glucoamylase was identified from a cDNA library using an anti-glucoamylase antibody as probe for product detection. When heterologously expressed in *S. cerevisiae* and *Kluyveromyces lactis* more than 90% of the synthesized glucoamylase was found to be secreted. The level of secreted enzyme was 20 times higher in *Kl. lactis* than that observed in *S. cerevisiae* transformants using a similar construct for transformation (Bui et al., 1996a, b).

In parallel biotechnologically important secretory enzymes like lipases were synthesized as recombinant proteins in *A. adeninivorans*. This temperature-sensitive protein with a pH - optimum at 7.5 hydrolyses ester bounds in triglycerides. Thereby fatty acids with middle-sized chains are more efficiently hydrolysed than those with short- or long-chains, with the highest activity on C8/C10 fatty acid esters *p*NP-caprylate, *p*NP-caprate and tricaprylin (Böer et al., 2005b).

The *AINV* gene provides another example of an interesting enzyme gene. The encoded invertase preferentially hydrolyzes -D-fructofuranosides and could be applied to the hydrolysis of sugar cane molasses or sugar beet molasses on an industrial scale. The *AINV* gene was obtained by screening a cDNA and a chromosomal library with a PCR amplificate corresponding to a particular gene segment. The isolated gene was expressed in recombinant *A. adeninivorans* strains fusing the coding sequence to the strong constitutive *TEF1* promoter. The resulting transformants were found to secrete the enzyme in high concentrations independent of the carbon source used for cultivation (Böer et al., 2004b).

An example for an interesting intracellular protein is a temperature-sensitive xylitol dehydrogenase which oxidizes polyols like xylitol and D-sorbitol and reduces simultaneously D-xylulose, D-ribulose and L-sorbose. Due to its optima at low temperatures and weak basic pH values the enzyme is of potential interest for application to food manufacturing processes. The respective *AXDH* gene was isolated and overexpressed in *A. adeninivorans* (Böer et al., 2005c).

A last example is the *ATAL* gene encoding a temperature-sensitive transaldolase with an acidic pH optimum. The preferred substrates for the enzyme include D-erythrose-4-phosphate and D-fructose-6-phosphate. Based on these properties the enzyme could be applied to C-C bounding and enantio-specific synthesis of novel sugars, as previously demonstrated for the *S. cerevisiae*-derived transaldolase (El Fiki et al., 2007).

27.5 The *A. adeninivorans*-Based Platform

27.5.1 Transformation System

A. adeninivorans provides an attractive novel gene expression platform. A first transformation system based on this dimorphic yeast species has been established more than a decade ago using *S. cerevisiae* and *A. adeninivorans*-derived *LYS2* genes for selection (Kunze et al., 1990; Kunze and Kunze, 1996). In these instances, transformation vectors either integrated into the chromosomes as single copy or in low copy numbers or were of episomal fate.

Improvements of the transformation system were introduced by Rösel and Kunze (1998) using a vector type (pAL-HPH1) that employed an rDNA targeting sequence for stable integration and the *E. coli*-derived *hph* gene (conferring hygromycin B resistance) under the control of the *A. adeninivorans*-derived *TEF1* promoter for dominant selection. As rDNA targeting sequence a 25S rDNA fragment from *A. adeninivorans* was used. The resulting hygromycin B-resistant transformants were found to harbour 2–10 plasmid copies stably integrated into the ribosomal DNA (Fig. 27.4a).

Employment of the dominant marker gene results in the undesired need for toxic compounds or antibiotics during the strain development. This can be avoided using auxotrophic strains and the respective gene sequence for complementation. As such the *AILV1* and *ALEU2* genes were isolated as described before and the respective auxotrophic strains were selected. In several examples the *A. adeninivorans ailv1* or *aleu2* hosts were transformed with the plasmids pAL-AILV1 containing the *AILV1* gene and pAL-ALEU2m containing the *ALEU2* gene for complementation (Fig. 27.4b and c). Transformants generated in this way were found to harbor 1–3 copies of the heterologous DNA mitotically stable integrated into the rDNA unit by homologous recombination (Steinborn et al., 2005; Wartmann et al., 1998, 2003b).

The *ailv1* and *aleu2* mutant strains selected after *N*-methyl-*N*Í-nitro-*N*-nitrosoguanidine treatment reverted to leucine/isoleucine prototrophy at a frequency of 10^{-6} (Samsonova et al., 1989, 1996). To eliminate this disadvantage, a *Δ atrp1* gene disruption mutant was generated. For disruption an amplified DNA fragment containing the *ALEU2m* gene flanked by *ATRP1* gene sequences of some 750 bp was employed (Fig. 27.5).

The generated auxotrophic host strain *A. adeninivorans* G1212 [*aleu2 atrp1:: ALEU2*] excels by mitotic stability during cultivation in both rich and minimal medium. The strain was transformed with the plasmid pAL-ATRP1, which contains the *ATRP1* gene as selection marker and for targeting the 25S rDNA. The resulting transformants contained a single chromosomal copy of the pAL-ATRP1 DNA (Steinborn et al., 2007b;– Fig. 27.4d).

For further platform improvement a novel vector element has been constructed that provides multicopy integration in *A. adeninivorans* G1212 [*aleu2 atrp1:: ALEU2*]. The element consists of the *ATRP1* coding sequence under control of a newly generated truncated *ALEU2* promoter of 53 bp. In several examples 8 and

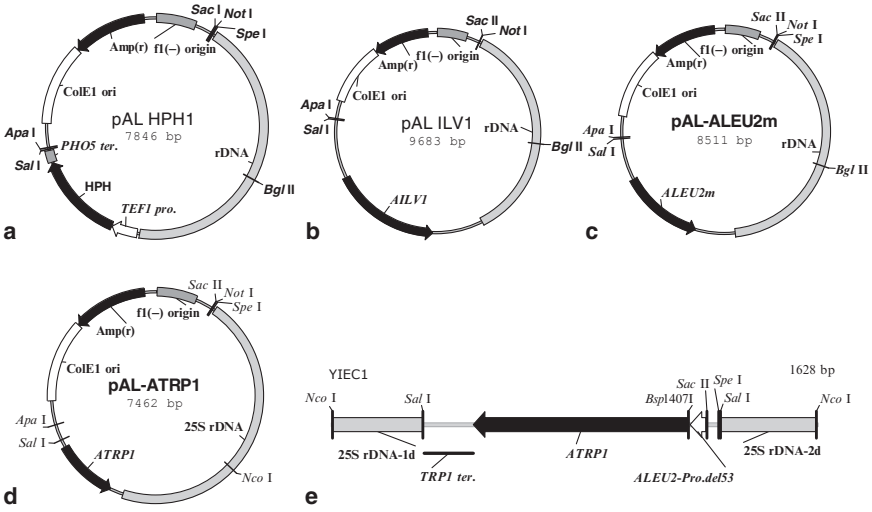


Fig. 27.4 Physical maps of vectors for the *A. adeninivorans*-based expression platform. (**a**) The vector pAL-HPH1 contains the following elements: a 25S rDNA sequence (rDNA) for chromosomal targeting, an expression cassette for the *E. coli*-derived *hph* gene in the order *A. adeninivorans*-derived *TEF1* promoter (TEF1 pro.), the *hph*-coding sequence (HPH), *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter.). The vector further contains unique *Apa*I and *Sal*I restriction sites for the insertion of the expression cassettes and an unique *Bgl*II site within the rDNA sequence for linearization. The vectors (**b**) pAL-AILV1, (**c**) pAL-ALEU2m and (**d**) pAL-ATRP1 contains the selection marker *AILV1* (AILV1), *ALEU2m* (ALEU2m) or *ATRP1* (ATRP1) instead of the expression cassette for the *E. coli*-derived *hph* gene. (**e**) Yeast integration-expression cassettes (YIEC1), a novel vector type for multicopy transformation of *A. adeninivorans* lacking an *E. coli* part. The YIEC1 flanked by *Nco*I sites contains the 25S rDNA sequences and the selection marker *ATRP1* fused to the deleted 58 bp *ALEU2* promoter

more copies of the vector were now found to be integrated in the genome of the recombinant strains instead of a single copy. In addition the vector design enables the integration of a small vector fragment that consists of yeast DNA only (yeast integration-expression cassette - YIEC) providing high transformation frequencies and a high mitotic stability (Steinborn et al., 2007a).

27.5.2 Heterologous Gene Expression

The construction of expression plasmids follows a two-step cloning strategy. First the heterologous genes are inserted between the respective *A. adeninivorans*-derived promoter and fungal terminator elements like *PHO5* from *S. cerevisiae* and *trpC* from *Aspergillus nidulans*. Subsequently the resulting expression cassettes (*A. adeninivorans* promoter – heterologous gene - fungal terminator) are integrated

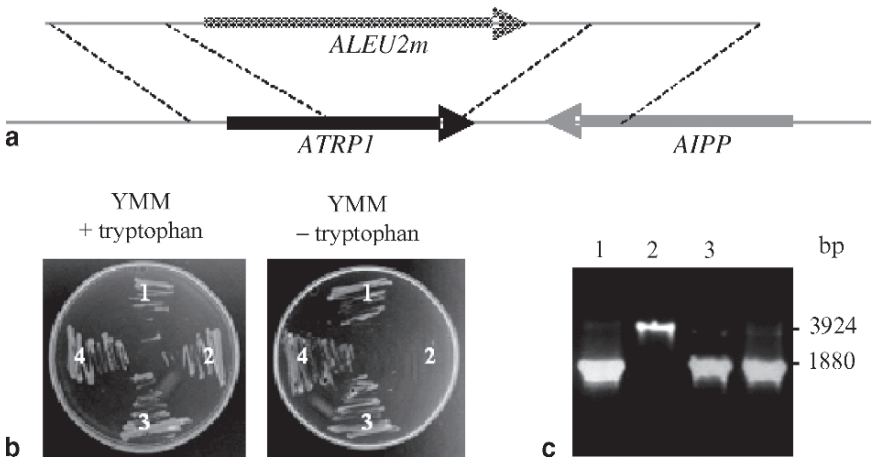


Fig. 27.5 *ATRP1* gene disruption and analysis of the *Δatrp1* mutants (**a**) The *ATRP1* gene replacement strategy by gene disruption. The *Δatrp1* fragment with 789 bp 5′-*atrp1* region – *ALEU2m* gene - 948 bp 3′-*atrp1* region was amplified by PCR and used to transform *A. adeninivorans* strain G1211 for sequential disruption of the chromosomal *ATRP1* copy. For this purpose the transformants were first selected for leucine prototrophy, second for tryptophan auxotrophy. (**b** and **c**) Analysis of the selected leucine-prototroph transformants for tryptophan auxotrophy and correct integration of the *Δatrp1* DNA fragment. *A. adeninivorans* G1211 (1) and the selected transformants (2–4) were spotted onto solid agar plates of YMM with and without tryptophan. The plates were incubated for 3 days at 30°C prior to photographic documentation. In parallel the genomic DNA of the analysed strains was isolated and used as template for amplification of the 3924 bp fragment with 5′-*atrp1* region – *ALEU2m* gene - 948 bp 3′-*atrp1* region with the primers ATRP1-1 and ATRP1-2

into the respective *A. adeninivorans* expression plasmid. For this purpose the cassettes are flanked by unique restriction sites (*Apa*I – *Sal*I, *Apa*I – *Xho*I, *Spe*I – *Sac*II, *Spe*I – *Not*I) allowing a directional integration (Fig. 27.4).

An increasing number of heterologous genes have been expressed in *A. adeninivorans*. As a first example the *XylE* gene from *Pseudomonas putida* encoding the catechol-2,3-dioxygenase was expressed under control of the *AILV1* promoter (Kunze et al., 1990; Kunze and Kunze, 1996; Table 27.5), followed by successful examples of genes expressed under control of the strong constitutive *TEF1* promoter. As such *GFP* and *HSA* gene sequences were inserted into the vectors pAL-HPH1 and pAL-ALEU2m and used to transform *A. adeninivorans* wild type and mutant strains. The recombinant strains contained 1–2 copies of the heterologous DNA integrated in 25S rDNA region. In case of *GFP* expression the recombinant protein was localized in the cytoplasm rendering the cells fluorescent. In case of *HSA*, the expression based on an ORF including the native 5′-signal sequence. Accordingly, the recombinant HSA was secreted to more than 95% into the culture medium. In 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.

Table 27.4 Isolated and sequenced genes of the yeast *A. adeninivorans* LS3

Gene	Gene product	Accession no.	Reference
AEFG1	Mitochondrial elongation factor G	AJ312230	(Wartmann et al., 2001)
AFET3	Copper-dependent Fe(II) oxidase	AJ277833	(Wartmann et al., 2002b)
AHOG1	Mitogen-activated protein kinase	AJ626723	(Böer et al., 2004a)
AHSB4	Histone H4	AJ535732	(Wartmann et al., 2003a)
AILV1	Threonine deaminase	AJ222772	(Wartmann et al., 1998)
AINV	β-Fructofuranoside fructohydrolase	AJ580825	(Böer et al., 2004b)
ALEU2	β-Isopropylmalate dehydrogenase	AJ488496	(Wartmann et al., 2003b)
ALIP1	Lipase	AJ879165	(Böer et al., 2005b)
ALYS2	Amino-adipate reductase	Not sequenced	(Kunze and Kunze, 1996)
APHO1	Acid phosphatase	AM231307	(Kaur et al., 2007)
ARFC3	Replication factor C component	AJ007712	(Stoltenburg et al., 1999)
ATAL	Transaldolase	AM400899	(El Fiki et al., 2007)
ATRP1	Phosphoribosyl anthranilate iso-merase	AM261500	(Steinborn et al., 2007b)
AXDH	Xylitol dehydrogenase	AJ748124	(Böer et al., 2005c)
GAA	Glucoamylase	Z46901	(Bui et al., 1996a)
TEF1	Elongation factor 1	Z47379	(Rösel and Kunze, 1995)
25S rDNA	25S rRNA	Z50840	(Rösel and Kunze, 1996)
18S rDNA	18S rRNA	Z50840	(Steinborn et al., 2005)
5.8S rDNA	5.8S rRNA	Z50840	(Steinborn et al., 2005)

Budding cells as well as mycelia secreted similar levels demonstrating a morphology-independent productivity (Wartmann and Kunze, 2003; Wartmann et al., 2003b; Table 27.5).

In addition to the *TEF1* promoter the strong constitutive *AHSB4* promoter was successfully assessed for suitability and was found to elicit similar expression levels (Wartmann et al., 2003a; Table 27.5).

Since *A. adeninivorans* is a dimorphic yeast, recombinant proteins can be produced in cells of different morphological stages. A *MF 1-IL6* fusion was expressed under control of the strong *TEF1* promoter in *A. adeninivorans* budding cells and mycelia. In contrast to other yeast species (*S. cerevisiae, H. polymorpha*) the recombinant interleukin-6 (IL-6) was correctly processed from the MF 1-IL6 precursor and was accumulated to more than 95% in the culture medium. In cultivation on a shaking flask scale a productivity of ca. 210 mg l^{-1} was observed in budding cell cultures and 145 mg l^{-1} in mycelial cultures (Böer et al., 2007; Fig. 27.6).

An approach to introduce simultaneously several genes was taken to established new metabolic pathways in *A. adeninivorans*. As an example the genes *phb*A, *phb*B and *phb*C of the polyhydroxyalkanoate (PHA) biosynthetic pathway of *Ralstonia eutropha* encoding -ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHA synthase were introduced for construction of a recombinant biocatalyst. *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

Table 27.5 Examples of heterologous gene expression in *A. adeninivorans* LS3

Gene	Donor organism	Gene product	Promoter	Vector	Recombinant protein level	Reference
lacZ	*E. coli*	β-Galactosidase	*GAA*	pAL-HPH1	350 kU mg^{-1}	(Wartmann and Kunze, 2000)
lacZ	*E. coli*	β-Galactosidase	*AHOG1*	pAL-HPH1	350 U mg^{-1}	(Böer et al., 2004a)
GFP	*Aequorea victoria*	Green fluorescent protein	*TEF1*	pAL-HPH1	n.d	(Wartmann et al., 2002a)
GFP	*Aequorea victoria*	Green fluorescent protein	*TEF1*	pAL-ALEU2m	n.d	(Wartmann et al., 2003b)
GFP	*Aequorea victoria*	Green fluorescent protein	*AHSB4*	pAL-ALEU2m	n.d	(Wartmann et al., 2003a)
HAS	*Homo sapien*	Human serum albumin	*TEF1*	pAL-HPH1	50 mg l^{-1}	(Wartmann et al., 2002a)
HAS	*Homo sapiens*	Human serum albumin	*TEF1*	pAL-ALEU2m	50 mg l^{-1}	(Wartmann et al., 2003b)
HAS	*Homo sapiens*	Human serum albumin	*AHSB4*	pAL-ALEU2m	50 mg l^{-1}	(Wartmann et al., 2003a)
XylE	*Ps. putida*	Catechol 2,3-dioxygenase	*AILV1*	I1-ALYS2	0.4 pkat mg^{-1}	
XylE	*Ps. putida*	Catechol 2,3-dioxygenase	*AINV*	pAL-ALEU2m	4.5 pkat mg^{-1}	(Böer et al., 2004b)
AINV	*A. adeninivorans*	Invertase	*TEF1*	pAL-ALEU2m	500 μkat l^{-1}	(Böer et al., 2004b)
PhbA	*R. eutropha*	β-Ketothiolase	*TEF1*	pAL-HPH1	2.2% PHA*	(Terentiev et al., 2004)
PhbB	*R. eutropha*	Cetoacetyl CoA reductase	*TEF1*	pAL-HPH1	2.2% PHA*	(Terentiev et al., 2004)
PhbC	*R. eutropha*	PHA synthase	*TEF1*	pAL-ALEU2m	2.2% PHA*	(Terentiev et al., 2004)
PhbC	*R. eutropha*	PHA synthase	*TEF1*	pAL-HPH1	2.2% PHA*	(Terentiev et al., 2004)
GFP	*Aequorea victoria*	Green fluorescent protein	*AXDH*	pAL-HPH1	n.d	(Böer et al., 2005c)
AXDH	*A. adeninivorans*	Xylitol dehydrogenase	*TEF1*	pAL-ALEU2m	900 mkat l^{-1}	(Böer et al., 2005c)
ALIP1	*A. adeninivorans*	Lipase	*TEF1*	pAL-ALEU2m	3,300 U l^{-1}	(Böer et al., 2005b)
amyA	*B. amyloliquefac.*	α-Amylase	*TEF1*	pAL-ALEU2m	150 μkat l^{-1}	(Steinborn et al., 2005)
hERα	*Homo sapiens*	Estrogene receptor α	*TEF1*	pAL-HPH1	n.d.	(Hahn et al., 2006)
phyK	*Klebsiella sp.ASR1*	Extracellular phytase	*GAA*	pAL-ALEU2m	75 mkat l^{-1}	(Hahn et al., 2006)
amyA	*B. amyloliquefac.*	α-Amylase	*TEF1*	pAL-ATRP1	300 μkat l^{-1}	(Steinborn et al., 2007b)
APHO1	*A. adeninivorans*	Acid phosphatase	*TEF1*	pAL-ALEU2m	70 mkat l^{-1}	(Kaur et al., 2007)
ATAL	*A. adeninivorans*	Transaldolase	*TEF1*	pAL-ALEU2m	35 mkat l^{-1}	(El Fiki et al., 2007)
HSA	*Homo sapiens*	Human serum albumin	*ATAL*	pAL-ALEU2m	0.6 mg l^{-1}	(El Fiki et al., 2007)
IL6	*Homo sapiens*	Interleukin-6	*TEF1*	pAL-ALEU2m	220 mg l^{-1} (b.c.) 145 mg l^{-1} (m.)	(Böer et al., 2007) (Böer et al., 2007)

*) % final product per dry weight n.d.) not detected b.c.) budding cell culture μ.) mycelial culture

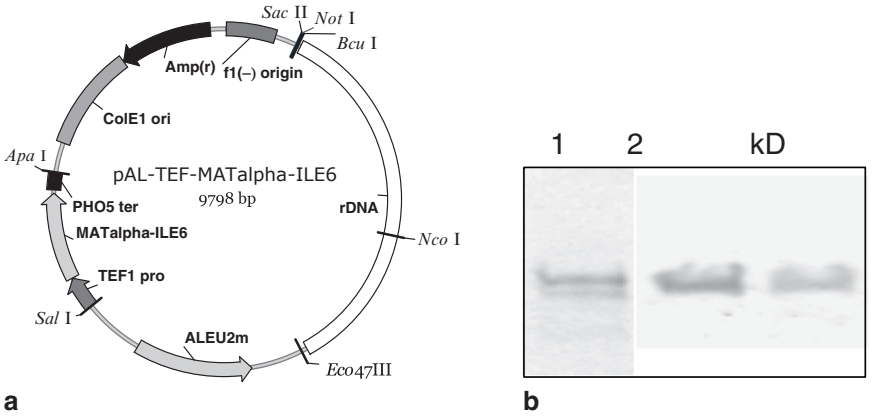


Fig. 27.6 (**a**) Physical map of the expression/integration vector pAL-ALEU2m-TEF-MATalpha-IL6. The vector contains the 25S rDNA sequence of *A. adeninivorans* (rDNA, white box), the selection marker *ALEU2m* (grey segment) and an expression cassette for the *IL6* gene in the order *A. adeninivorans*-derived *TEF1* promoter (TEF1 pro, grey segment), the *IL6*-coding sequence, *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter, black bar) as selection marker. The vector contains a unique *Nco*I site for linearization within the rDNA sequence (**b**) IL6 accumulation in recombinant *A. adeninivorans* budding cell and mycelial cultures. The strains were cultured in YMM supplemented with 2% glucose for 72 h at 30°C (budding cells) or 45°C (mycelia). 20 µl aliquots of culture media were separated on SDS-PAGE (11%) gels, transferred to nitrocellulose filters and probed with anti-IL-6 antibodies. The concentration of recombinant IL-6 was calculated from the signal intensity of an IL-6 standard. (1) IL-6 standard (*E. coli*), (2) *A. adeninivorans* G1211/pAL-ALEU2m-TEF-MATα-IL6 – budding cells (205 mg l^{-1}), (3) *A. adeninivorans* G1211/pAL-ALEU2m-TEF-MATα-IL6 – mycelia (144 mg l^{-1}) (**c**) N-terminus of IL-6 secreted from recombinant *A. adeninivorans* (budding cell and mycelial cultures).genes was able to accumulate up to 2.2% (w/w) PHV and 0.019% (w/w)

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 containing all three *phb* PHB (Terentiev et al., 2004; Fig. 27.6 and 27.7).

The *A. adeninivorans* transformation/ expression system can be used for promoter assessment. For this purpose the *lacZ* gene from *E. coli,* the *GFP* gene from *Aequorea victoria,* the *phyK* gene from *Klebsiella* spec. and the *XylE* gene from *Ps. putida* can be employed as reporter genes. In previous examples the expression cassettes containing the *GAA, AHOG1, AINV, AXDH* as well as the *ATAL* promoter – reporter gene – *PHO5* terminator were analyzed. The characteristics of the selected promoters could be assessed for aspects like dependence on carbon source, osmolarity of the medium or morphological stage (Böer et al., 2004a,

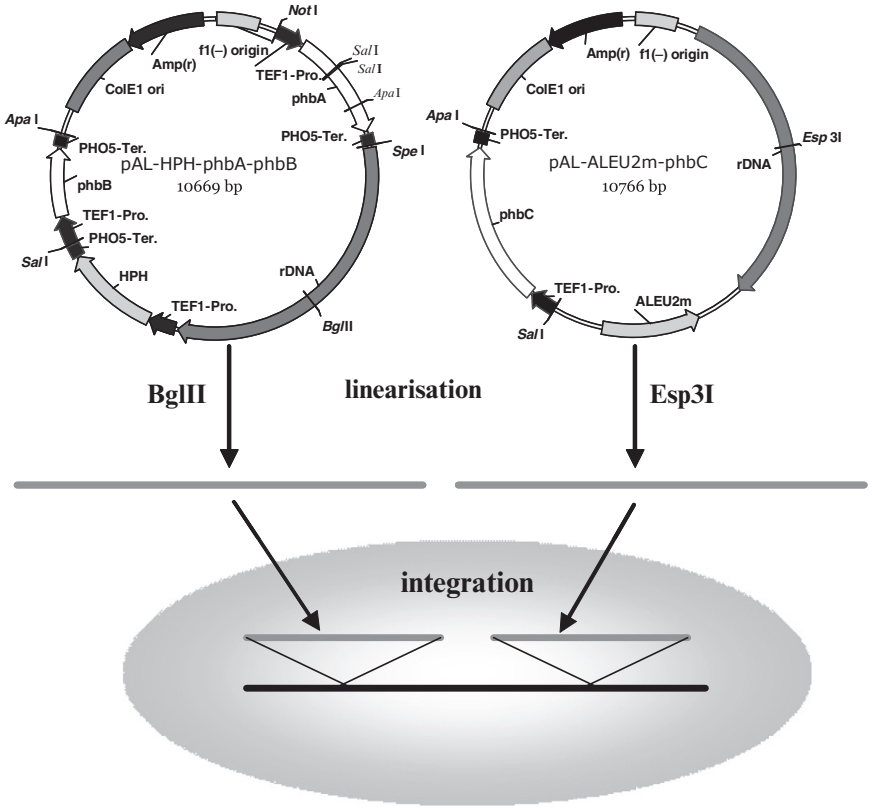


Fig. 27.7 Transformation procedure based on simultaneous integration of the plasmids pAL-HPH-phbA-phbB and pAL-ALEU2m-phbC into the 25S rDNA of *A. adeninivorans* G1211 (*aleu2*). The two plasmids pAL-HPH-phbA-phbB and pAL-ALEU2m-phbC containing the expression cassettes with *phbA, phbB* and *phbC* genes 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 (plasmid pAL-HPH-phbA-phbB) or the complementation of the *aleu2* mutation (plasmid pAL-ALEU2m-phbC)

2004b, 2005c; El Fiki et al., 2007; Hahn et al., 2006; Wartmann and Kunze, 2000; Table 27.5).

As another application of recombinant strains a novel estrogen biosensor has been developed. For this purpose, recombinant *A. adeninivorans* strains were engineered co-expressing the human estrogen receptor (hER) and a *Klebsiella*-derived phytase (*phyK*) reporter gene under control of an *A. adeninivorans*-derived glucoamylase (*GAA*) promoter modified by insertion of estrogen-responsive elements (EREs). In response to the presence of estrogenic compounds, two estrogen-hER complexes dimerize and bind to estrogen-responsive elements (ERE)

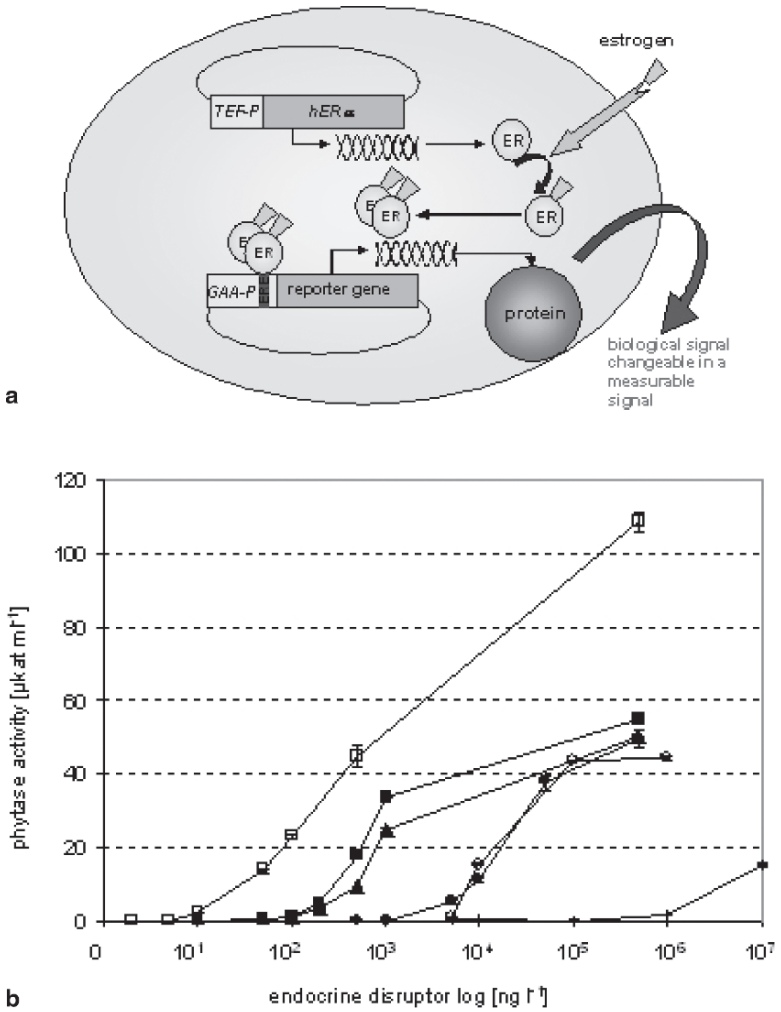


Fig. 27.8 (**a**) Principle of an estrogen sensor based on recombinant *A. adeninivorans* strains (A-YES). *A. adeninivorans* G1211 transformed with the plasmids pAL-HPH-hERα and pAL-ALEU2m-GAA(2xERE-107)-phyK (G1211/pAL-HPH-hERα - pAL-ALEU2m-GAA(2xERE-107)-phyK) is the bio-component of the A-YES. It expresses the estrogen receptor gene (*TEF1* promoter – *hERα* gene – *PHO5* – terminator) constitutively and produces a relatively constant level of recombinant hERα independent of the estrogen concentration. In the presence of estrogen or estrogen analogues, however, hERα forms a hERα-estrogen-dimer complex, which binds to the ERE-region of the *GAA* promoter located in the second reporter gene expression cassette. The cassette (*GAA-ERE* – promoter – *phyK* gene – *PHO5* terminator) is activated, the *phyK* gene is expressed and phytase is synthesized. Since this enzyme contains a native signal sequence it is secreted and accumulates extracellularly. The recombinant phytase level can then be quantified using a simple biochemical method. (**b**) Specificity of the A-YES based on *A. adeninivorans* G1211/pAL-HPH-hERα - pAL-ALEU2m-GAA(2xERE-107)-phyK for a range of steroids and steroid metabolites. The graphs depict the log concentration of 17α-ethynylestradiol (α), 17 -estradiol (■), estrone (▲), estriol (●), coumestrol (○) and bisphenol A (+) plotted against the recombinant phytase activity of the medium after 30 h incubation

within the promoter to subsequently induce the expression of the reporter gene. The insertion of different numbers of EREs in three alternative positions within the promoter and its effect on reporter gene expression were assessed. In a particular construct, a detection limit of 5 ng l^{-1} and a quantification limit of 10 ng l^{-1} for 17 -estradiol-like activity could be achieved. A convenient photometric assay enables estrogen monitoring in sewage samples within 30 hrs (Hahn et al., 2006; Fig. 27.8).

27.6 Conclusions and Perspectives

A. adeninivorans is a haploid, dimorphic, non-pathogenic, ascomycetous, anamorphic, arthroconidial yeast. It is an attractive organism for both, basic and applied research. The very broad range of substrates which can be used as carbon and/or nitrogen sources, the growth and secretion characteristics, the thermo- and osmotolerance as well as the temperature-dependent dimorphism make this yeast an attractive organism for biotechnological application. *A. adeninivorans* is an interesting host for the synthesis of special products because all essential prerequisites and components for heterologous gene expression are available. In addition, the exceptional properties make *A. adeninivorans* a potential donor for genes underlying such properties to equip traditional biotechnologically applied organisms with new attractive capabilities.

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