Chapter 18
Advances in Gene Expression in Non-Conventional Yeasts

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Contents

Abstract Yeast has been a favoured lower eukaryotic system for the expression and production of recombinant proteins for both basic research and practical applications, and the demand for foreign-gene expression systems is increasing rapidly. Despite the vast amount of information on the molecular biology and physiology of *Saccharomyces cerevisiae*, which has consequently been the first choice as host

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system for recombinant protein production in the past, several limitations have been identified in this expression system. These limitations have recently been relieved by the development of expression systems in other yeast species known as 'non-conventional yeasts' or 'non-*Saccharomyces*' yeasts. With the increasing interest in the biotechnological applications of these yeasts in applied and fundamental studies and processes, the term 'non-conventional' yeast may well soon become redundant. As there is no universal expression system for heterologous protein production, it is necessary to recognize the merits and demerits of each system in order to make a right choice. This chapter will evaluate the competitive environment of non-conventional expression platforms represented by some of the best-known alternative yeasts systems including *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Hansenula polymorpha*, *Pichia pastoris* and more recently, *Arxula adeninivorans*.

Keywords Recombinant proteins, expression systems, non-conventional yeast, heterologous protein, expression platforms

18.1 Yeasts as Eukaryotic Expression Systems

As large numbers of new genes become available due to the progress of various genome projects, methods for highly efficient expression and production of heterologous proteins, for both industrial and academic purposes, are currently under intense investigation.

Gerngross (2004) summarized five primary parameters for evaluating protein expression platforms:

- The cost of manufacturing and purification,
- The ability to control the final product, including its post-translational processing,
- The time required from gene to protein,
- The regulatory path to approve a drug/protein produced on a given expression platform,
- The overall royalties associated with the production of a recombinant product in a given host.

Yeasts are unicellular eukaryotic organisms and have been used for the expression of a wide variety of heterologous proteins from diverse origins. This popularity is due to the fact that it combines the advantages of bacterial expression systems in terms of ease of manipulation and established fermentation technologies with the ability to perform eukaryotic processing of polypeptides expressed by these systems (Gellissen and Hollenberg, 1997). Unlike higher eukaryotic cell lines producing recombinant proteins, yeasts can produce high protein titers (up to 14.8 g l^{-1}) growing on relatively inexpensive chemically defined media (free from animal derived supplements) in a short fermentation process (lasting only a few days) that is easily scalable to 100 m^3, thereby yielding a rapid turnaround from gene to protein (Gerngross, 2004).

The emerging of protein-based therapeutics as the largest class of new chemical entities being developed by drug companies (Walsh, 2003), lead to renewed interest in the production of these therapeutics by systems that could be engineered to produce recombinant proteins exhibiting properties similar to native proteins. One of these properties is the correct glycosylation of the therapeutic protein to allow the recombinant protein to be therapeutically equally effective when compared to the native counterpart. Yeasts have the ability to glycosylate glycoproteins, but unfortunately *N*-glycosylation in yeast is generally coupled with hypermannosylation of the core oligosaccharides (Gerngross, 2004), yielding proteins with suboptimal folding, function and stability in human serum (Helenius and Aebi, 2001). Fortunately, the abundance of molecular biology tools and techniques available in yeasts made it possible for researchers at GlycoFi Inc. (USA) and the Korean Research Institute of Bioscience and Biotechnology (KRIBB, Korea) to humanize the *N*-glycosylation patterns of glycoproteins produced by *Pichia pastoris* and *Hansenula polymorpha*, respectively (Wildt and Gerngross, 2005; Gellissen et al., 2005). Solving the non-human glycosylation problem would most possibly allow yeast to be one of the major hosts systems for the production of overall therapeutic proteins.

Another problem, occurring when a host was used for the heterologous expression of foreign proteins, was the difference in codon bias and usage between the donor organism and the recipient host. The non-codon optimized gene expression resulted in an apparent poor expression record for several host strains that exhibited a different in codon bias compared to the foreign gene being expressed (Yadava and Ockenhouse, 2003; Gustafsson et al., 2004). Recent advances in the quality as well as the reduced production costs of oligonucleotide synthesis allowed for the artificial synthesis of genes and even complete genomes (Smith et al., 2003). This artificial synthesis, together with codon preference tables available on the internet (http://www.kazusa.or.jp/codon/), allows for custom designed, host codon optimized genes – an approach exploited by several commercial companies (GENEART, Germany; Codon Devices, USA), that provides a cost-effective (from US$ 0.79/bp), rapid turnaround service to the biotechnology community.

In this chapter, we will briefly highlight the latest developments in *Saccharomyces cerevisiae* followed by a more comprehensive analysis of the available expression systems in the most widely used non-conventional yeasts. These are *Arxula adeninivorans, Kluyveromyces lactis, Yarrowia lipolytica, Hansenula polymorpha* (*Pichia angusta)* and *Pichia pastoris*. We also briefly discuss two additional yeast species (*Schwanniomyces occidentalis* and *Pichia stipitis)* which, due to specific properties, have the potential to also become widely used in heterologous expression.

18.2 Saccharomyces cerevisiae

Saccharomyces cerevisiae has been used for thousands of years for the purpose of brewing and baking and has been granted GRAS (generally recognized as safe) status. An overwhelming wealth of information on genetics, molecular biology and

physiology has been accumulated on this organism (Rose and Harrison, 1989; Broach et al., 1991), making this traditional species the best-characterized eukaryotic system today (Watson et al., 1987). Several limitations, in terms of using *S. cerevisiae* as a tool for heterologous production, have been reported in the past (Buckholz and Gleeson, 1991). These included reduced biomass yield due to aerobic alcohol fermentation, very low yields (with a maximum of 1–5% of total protein), hyperglycosylation, plasmid instability, and the retention of protein in the periplasmic space. It is due to these limitations that attention was redirected to non-*Saccharomyces* or non-conventional yeasts as hosts for heterologous production of proteins.

Researchers have, however, not abandoned *S. cerevisiae* due to these limitations, but invented strategies to overcome most of these limitations, thereby making *S. cerevisiae* the most utilized yeast system for the production of biopharmaceutical products (Graumann and Premstaller, 2006). Researchers at Novozyme Delta Ltd. in the UK (previously Delta Biotechnology Ltd. before being acquired by Novozymes) solved the yield problem, by producing recombinant proteins of up to 40% (w/v) total intracellular protein, or secreting recombinant proteins up to 5.5 g l^{-1}. To obtain these values, the native 2 μm plasmid had to be engineered to be stably propagated under non-selective conditions with elevated copy numbers (copy number was increase from 60 to 100 copies per genome), together with an additional multiple cloning site in the vector for co-expression of an additional gene (Sleep et al., 2001; C. Finnis, personal communication). A recent comprehensive review of approved biopharmaceuticals in the United States and/or Europe indicated that 24% of approved biopharmaceutical products were expressed in *S. cerevisiae* (Fig. 18.1) (Melmer, 2005), even though hyperglycosylation of glycoproteins still presents a major obstacle in the use of *S. cerevisiae* in therapeutic protein production. The reason for this is that the ratio of glycosylated proteins to non-glycosylated

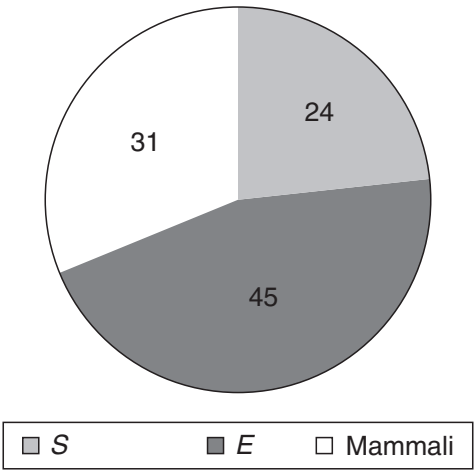

Fig. 18.1 Chart indicating the percentage of biopharmaceutical products (approved in the US and/or Europe) produced in the three most popular expressions host platforms (Melmer, 2005)

proteins for therapeutic use is 60:40, allowing the theoretical use of hosts such as *S. cerevisiae* and *Escherichia coli* for the production of 40% of the therapeutic protein market (Gerngross, 2004).

The deciphered genome of *S. cerevisiae*, together with the myriad of tools and techniques developed for manipulating and dissecting gene function in *S. cerevisiae*, allowed several scientists to move beyond normal recombinant protein expression studies (Goffeau et al., 1996). Recent literature reported on the engineering and manipulation of complex metabolic pathways to allow the accumulation or over-production of pathway intermediates, even improving process performance and cellular properties, again exploring the unknown for others to follow (Wada et al., 2006; Ostergaard et al., 2000; Mutka et al., 2006; Ro et al., 2006).

18.3 Arxula adeninivorans

18.3.1 History

Arxula adeninivorans is a non-pathogenic, xerotolerant, ascomycetous, anamorphic, arthroconidial yeast which was first known as *Trichosporon adeninovorans* (Middelhoven et al., 1984). In 1990 it was renamed as *Arxula adeninivorans (*van der Walt et al., 1990). The genus *Arxula* consists of two species, *Arxula terrestre* [(van der Walt and Johanssen) van der Walt, Smith and Yamada (1990)], which is the type species of the genus, and *A. adeninivorans* [(Middelhoven, Hoogkamer-Te Niet and Kreger van Rij) van der Walt, Smith and Yammada (1990)].

18.3.2 Introduction

Middelhoven et al. (1984, 1991, 1992); van der Walt et al. (1990) and Gienow et al. (1990) demonstrated that this yeast species is able to assimilate and ferment many compounds as the sole source of carbon and energy. Both *Arxula* species, *A. adeninivorans* and *A. terrestre*, are conspicuous due to their utilization of nitrate. They are able to grow on adenine, uric acid, butylamine, pentylamine or putrescine as sole source of carbon and energy (Wartmann and Kunze, 2000). However, *A. terrestre*, in contrast to *A. adeninivorans*, has no fermentative ability and does not assimilate soluble starch, melibiose, melizitose, propylamine or hexylamine (Middelhoven et al., 1984). *A. adeninivorans* is able to use a wide range of sugars as substrates, which is enabled by the secretion of enzymes such as glucoamylase, acid phosphatase I and II, trehalase, cellobiase I and II, β-D-xylosidase, 3-phytase and invertase (Wartmann and Kunze, 2000). In addition, *A. adeninivorans* assimilates polyalcohols and organic acids used in the conventional carbon assimilation test, except for L-rhamnose, inulin, lactose, lactate and methanol. Likewise, all conventionally

used nitrogen compounds, except creatine and creatinine, are suitable nitrogen sources. Several nitrogen compounds, like amino acids and purine derivatives, many primary *n*-alkyl-amines and terminal diamines, are metabolized as sole carbon, nitrogen and energy sources. In the case of alcohols, dialcohols, carboxylic acids, dicarboxylic acids and other nitrogen-less analogous compounds and intermediates of the general metabolism are also assimilated. In addition, *A. adeninivorans* also degrades some phenols and hydroxybenzoates (Gienow et al., 1990; Middelhoven et al., 1991, 1992).

Not only can *A. adeninivorans* grow on many different carbon sources, but produces and secretes several extracellular enzymes into the culture medium during cultivation. Besides RNAse, some proteases, various glucosidases such as glucoamylase, β-glucosidases, pectinases, xylosidase, some acid phosphatases, trehalose, some cellobiose, invertase and phytase could be detected (reviewed by Wartmann and Kunze, 2000).

Furthermore, an interesting property of *A. adeninivorans* is its osmotolerance. This yeast can grow in minimal as well as rich media containing up to 3.32 osmomol kg^{-1} water in the presence of ionic (up to 20% NaCl), osmotic (PEG400) and water stress (ethylene glycol) (Gellissen et al., 2005). The influence of NaCl concentration of up to 10% NaCl on growth parameters is weak, but NaCl concentrations of higher than 10% cause a decrease in the specific growth rate, a longer adaptation phase and a lower cell count during the stationary growth phase (Yang et al., 2000). In addition to the tolerant behaviour of *A. adeninivorans* in high-salt conditions, results obtained by Wartmann and co-workers (2003a) demonstrated that the *A. adeninivorans AHSB4* gene (encoding histone H4) is strongly expressed in the presence and absence of NaCl. The maintenance of a strong expression profile under high-salt conditions is an unprecedented characteristic not reported for any other fungal histone H4 gene. Therefore, the *AHSB4* promoter is an attractive control element for heterologous gene expression, especially when defining fermentation condition with media of high osmolarities (up to 10% NaCl).

18.3.3 Arxula adeninivorans Strains

Besides the aforementioned properties, the temperature-dependent dimorphism of *A. adeninivorans* is of biotechnological interest. Dimorphism in fungi refers to two states of the same organism, namely budding cells and mycelia. It is a characteristic of several fungi (Shepherd, 1988) and can be influenced by changes in a variety of parameters as described by San Blas and San Blas (1984). Although the pathogenic yeast *Candida albicans* is generally used as a model organism in the analysis of dimorphism, the isolation of mutants and the genetic and molecular biological handling of *C. albicans* are difficult since it is an asexual diploid organism (Hubbard et al., 1986; Gil et al., 1990). The *A. adeninivorans* strain LS3 can grow at temperatures of up to 48°C without previous adaptation (Wartman et al., 1995). Wartmann et al. (1995) found that temperatures above 42°C induce a morphological transition from budding to mycelial form, concomitantly with an altered gene expression pattern.

This environmentally conditioned dimorphism is reversible, and budding is re-established when the cultivation temperature is decreased to below 42°C. This environmentally conditioned dimorphism in conjunction with the molecular biological characteristics (Gienow et al., 1990) and the haploidy of *A. adeninivorans* LS3 provide the possibility of selecting a large number of mutants (Samsonova et al., 1989, 1996). Temperature shift-induced dimorphism has already been described for the fungi *Histoplasma capsulatum* (Medoff et al., 1981; Marcesca and Kobayashi, 1989), *Blastomyces dermatitidis* (Burg and Smith, 1994) and *Paracoccidoides brasiliensis* (San Blas and San Blas, 1993). In contrast to *A. adeninivorans*, these fungi grow as mycelia at lower temperatures (25°C) and they form budding cells after an increase in the cultivation temperature to 37°C. Other well-characterized dimorphic yeasts with a temperature-dependant dimorphism, e.g. *C. albicans* and *Y. lipolytica*, need an additional factor to induce morphological shifts such as a change in pH or the addition of serum to the medium (Buffo et al., 1994; Orlowski, 1994; Saporito-Irwin et al., 1995). This is in contrast to *A. adeninivorans*, for which the temperature-regulated dimorphism is independent of the medium and the carbon source used (Wartmann et al., 1995). Because *A. adeninivorans* LS3 is non-pathogenic haploid yeast with many biotechnological important properties, the secretion behavior of budding cells and mycelia was analyzed in detail (Wartmann et al., 2000). In order to analyze whether the dimorphism observed in *A. adeninivorans* is exclusively influenced by high temperatures or also by other parameters, Wartman and coworkers (2000) selected, identified and characterized a large number of mutants with altered behaviour in dimorphism. These mutants formed mycelia at 30°C and therefore enabled the distinguishing of temperature-mediated and morphology-related effects on gene expression. The analysis of these mutants showed that most of the dimorphic mutants were not temperature-sensitive, since morphological changes did not correlate with changes in temperature. This indicates that morphology rather than temperature is the decisive factor in the analyzed process (Wartmann et al., 2000).

Protein secretion plays an important role in filamentous fungi. In particular, the high levels of native proteins secreted by fungi make these organisms excellent hosts for the expression of recombinant proteins. In addition, yeast species that form either budding cells or pseudomycelia, e.g. *S. cerevisiae*, *P. pastoris* and *H. polymorpha*, have also been used as hosts for heterologous gene expression (Sudbery, 1996; Gellissen and Hollenberg, 1997). Similarly, mycelial cultures of *A. adeninivorans* accumulate two-fold higher protein concentrations and contain two- to five-fold higher glucoamylase and invertase activities in the medium than budding cells. Cell morphology was also 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 characteristics 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 such as biological activity or immunological tolerance (Wartmann et al., 2002a).

18.3.4 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 (Samsonova et al., 1989; Gienow et al., 1990; Wartmann et al., 2000). The first transformation system for *A. adeninivorans* was developed by Kunze and Kunze (1996) using the *LYS2* genes from *A. adeninivorans* or *S. cerevisiae* as selective markers. A second transformation procedure was based on a dominant selection marker, such as the *E. coli hph* gene, which allows resistance against the toxic hygromycin B (Rösel and Kunze, 1998). To avoid the employment of an undesired dominant marker gene and the use of toxic compounds or antibiotics during strain development, auxotrophic strains and the respective gene sequences for complementation should be available. The haploidy of *A. adeninivorans* enabled the selection of mutants by *N*-methyl-*N*Î-nitro-*N*-nitrosoguanidine (NG) and UV mutagenesis. A large number of auxotrophic mutants and mutants with altered catabolite repression that were resistant to 2-deoxy-D-glucose were selected and characterized by Samsonova et al. (1989, 1996) and Büttner et al. (1989). These mutants are characterized by a high synthesis of some extracellular enzymes in the presence of different carbon sources.

Wartmann et al. (1998) described the *AILV1* gene encoding threonine deaminase as a selection marker for transformation of an *A. adeninivorans ailv1* mutant strain. Using the *A. adeninivorans*-derived 25S rDNA–targeting approach, recombinant strains were generated harboring one to three copies of the foreign DNA integrated into the 25S rDNA. However, this system suffered several drawbacks rendering it unsuitable for efficient heterologous gene expression. These drawbacks included that all identified *ailv1* mutant strains showed an atypical anamorphic phenotype causing an inefficient separation of the yeast transformants from the media after centrifugation. In addition, the large size of the transformation plasmid prevented simple integration of expression cassettes (Wartmann et al., 2003b). However, a range of auxotrophic *A. adeninivorans* mutants, showing a typical growth phenotype, have been identified (Klabunde et al., 2002), such as the *aleu2* mutant. Subsequently, Wartmann et al. (2003b) established a host-vector system based on complementation of this auxotrophy. The respective *ALEU2* gene was isolated and incorporated into suitable transformation vectors. For targeting, the plasmid additionally contained the *A. adeninivorans* 25S rDNA similar to, like the previously designed vector. However, a disadvantage of the *A. adeninivorans* platform is the low copy number of the heterologous DNA targeted to the 25S rDNA (Wartmann et al., 2002b, 2003b). This results in comparably low productivities, and results obtained by Wartmann and co-workers (2003a) confirmed a gene-dosage effect on heterologous gene expression. When strains were co-transformed with two rDNA integration vectors equipped with two different selection markers it resulted in two mitotically stably integrated copies, with transformants displaying a considerable increase in productivity. Use of defective promoters in the complementation gene may result in higher copy numbers. However, in all instances mitotic stability under non-selective conditions was found to be high. In previous studies (Wartmann et al., 2002b), based on the

plasmid pAL-HPH1, the cassette '*TEF1* promoter - heterologous gene - *PHO5* terminator' has already been successfully applied for heterologous gene expression. The *TEF1* promoter provides a strong and constitutive expression of a heterologous gene (Müller et al., 1998; Wartmann et al., 1998) even when present in low copy numbers. This was confirmed in a study by Wartmann et al. (2003a) using *GFP* and *HSA* as model genes. In this respect, the *A. adeninivorans* system differs from most other non-conventional yeast systems, in which higher copy numbers are required to obtain comparable high expression levels (Juretzek et al., 2001; Gatzke et al., 1995). Current developments by Wartmann and co-workers (2003a) are aimed at further increasing the copy number and thereby potentially improving the productivity of recombinant *A. adeninivorans* strains.

A. adeninivorans is a non-conventional yeast with a range of properties attractive for both basic and applied research. The very extensive range of substances that can be utilized as carbon and/or nitrogen sources, its growth and secretion behaviour, thermo- and halotolerance and temperature-dependent dimorphism makes this yeast an attractive organism for biotechnological research. In addition, it is an excellent host for heterologous gene expression and might replace or supplement other yeast-based systems.

18.4 Kluyveromyces lactis

18.4.1 History

Kluyveromyces lactis belongs to the family Saccharomycetaceae in the genera *Kluyveromyces* and consist of two varieties; *Kluyveromyces lactis* (Dombrowski) van der Walt var. *lactis* and *Kluyveromyces lactis* var. drosophilarum (Shehata, Mrak and Phaff) (Sidenberg and Lachance, 1986). The former is heterothallic and ferments lactose and the latter is homothallic and does not ferment or assimilate lactose (Lachance, 1998). Most of the published work that has been performed on *K. lactis,* was performed on the variety *lactis.* In the genera *Kluyveromyces* there are 15 accepted species (*Kluyveromyces* van der Walt emend. (van der Walt in Lachance (1998)). The key character for the genus *Kluyveromyces* is ascus deliquescence, however, (Kurtzman, 2003) showed that this character have no phylogenetic basis. Phylogenetic analysis based on 18S, 5.8S, ITS and 26S rDNAs, translation elongation factor 1-α, mitochondrial small-subunit rDNA and COXII showed that species of *Kluyveromyces* are found in six clades indicating the polyphyly of the genus as presently defined. It was also previously proposed that the six known species of the *K. marxianus* clade (including *K. lactis*) should be placed under the genus *Zygofabospora* (Naumov and Naumova, 2002). It was however proposed that changing the specie name of the biotechnological important species *K. lactis* and *K. marxianus* is inconsistent with nomenclature stability of well known species (Kurtzman et al., 2001; Kurtzman, 2003).

18.4.2 Introduction

K. lactis has been studied since the early 1960s. The natural habitat of this species is diverse, but many strains were originally isolated from milk-derived products in which the major carbon source is lactose. However, study of this yeast was performed in a far lesser extend in comparison to its closely related neighbor *S. cerevisiae*. In the 1980s more prominence was given to *K. lactis* due to the identification of a DNA plasmid based killer system, lactose metabolism and secretion of recombinant proteins.

K. lactis is amenable to genetic studies, having four spores in an evanescent ascus, so that single-spore cultures can be easily obtained. The ability of this yeast to secrete high molecular weight protein, specifically a killer toxin, increased the interest in the use of this yeast as a host for heterologous expressed proteins (Wésolowski-Louvel et al., 1996). Because of its distinctive physiological properties, *K. lactis* has become an important alternative to the classical *S. cerevisiae*. *K. lactis* is well known for its ability to produce β-galactosidase and as an expression host for the production of the milk clotting enzyme bovine chymosin (van den Berg et al., 1990). This yeast is also used to commercially produce the native enzyme lactase that is sold under the trade name Maxilact™ by DSM Food Specialties, Delft, The Netherlands (van Ooyen et al., 2006).

18.4.3 Kluyveromyces lactis Strains

Most of the initial work in the 1960s conducted on *K. lactis* was performed by the group of Harlyn O. Halvorson at Madison, Wisconsin (then called *Saccharomyces lactis*) (Fukuhara, 2006). The initial work was based using two isolates, a *MATa* mating type strain NRRL Y-1140 (CBS 2359) (the proposed reference strain for genetic and molecular studies; (Wésolowski-Louvel et al., 1996)) and a *MATα* mating type strain NRRL Y-1118 (CBS 6315). The major genetic background seems to come from these two strains together with the *MATα* strain NRRL Y-1205 (CBS 2360) (Wésolowski-Louvel et al., 1996; Fukuhara, 2006). The main industrial strain is a prototrophic wild-type isolate GG799 that has an excellent protein expression track record. The main advantage of this strain is the low amount of glucose repression of the *LAC4* promoter; therefore *LAC4*-based heterologous expression is high in media containing glucose as carbon source (van Ooyen et al., 2006).

18.4.4 Genetics and Molecular Biology

The complete genomic sequence of *K. lactis* has been determined through the Génolevures project. Strain CLIB 210 which is an auxotrophic derivative of strain CBS 2359/NRRL Y-114 was used as source for genomic DNA. The *K. lactis*

genome consists of approximately 10.6 Mb (not including the rDNA) and is organized as six chromosomes ranging in size from 1 to 3 Mb, containing 5329 open reading frames (Bolotin-Fukuhara et al., 2000; Dujon et al., 2004).

A pair of linear plasmids (pGKL1 and pGKL2) has been isolated in certain strains of *K. lactis* and confers a killer phenotype (Gunge, 1986; Gunge and Kitada, 1988; Volkert et al., 1989; Stark et al., 1990). pGKL1 is a 8.8 kb double-stranded DNA plasmid and contains the genes that encode the killer toxin subunit proteins. pGKL2 is a 13.4 kb plasmid and is needed for the maintenance of pGKL1. Attempts have been made to use these plasmids in heterologous expression; however, expression levels were low in comparison with results obtained when circular vectors were used.

There are no naturally-occurring circular plasmids in *K. lactis* (*Kluyveromyces lactis* var. *lactis*). Vectors derived from the 2μ plasmid of *S. cerevisiae* can be used to transform *K. lactis* strains, but are very unstable, requiring maintenance of selective pressure. The most widely used replicating vector system specific for *K. lactis* is based on the plasmid pKD1 isolated from *Kluyveromyces drosophilarum* (*Kluyveromyces lactis* var. *drosophilarum*) (Chen et al., 1986; Falcone et al., 1986). pKD1 belongs to the 2μ family plasmids sharing the same type of gene organization, although there is little homology of nucleotide sequence. Analysis of individual functional elements of pKD1 has also been described (Bianchi et al., 1991, 1992). This plasmid is maintained in the region of 60 to 80 copies per cell, but reduction in copy number to approximately 20 copies per cell have been observed when containing some heterologous genes (Falcone et al., 1986; Morlino et al., 1999). Instability of this plasmid expressing a human lysozyme was also shown by Iwata et al. (2004) where only 17% of cells retained the plasmid after growth in rich media (YPD) for 20 generations. At the same time an integrated expression vector was retained by more than 90% of the cells producing the same enzyme. Furthermore, a pKD1 based plasmid containing a *K. lactis* centromeric region was retained by at least 70% of transformed cells after 20 generations.

Transformation of *K. lactis* can be achieved by most standard methods (Iborra, 1993; Wésolowski-Louvel et al., 1996). The standard *S. cerevisiae* markers such as *TRP1, URA3* and *LEU2* genes have been conveniently used in *K. lactis*, because corresponding mutations in *K. lactis* can be complemented by these genes under their native promoter. Study of non-conventional yeasts often suffers from the absence of available auxotrophic mutants as transformation hosts. Sensitivity of many yeasts, including *K. lactis*, to the antibiotic G418 (geneticin) allows the use of the kanamycin resistance gene (coding for 3′-aminoglycoside-phosphotransferase) as a transformation marker (Sreekrishna et al., 1984). A selection marker free system based on acetamidase was developed and patented by Selton et al. (2000). Cells transformed with a vector system containing this marker gene are grown in media lacking nitrogen source (for example yeast carbon base) but supplemented with acetamide. Expression of acetamidase converts acetamide to ammonia which acts as a source of nitrogen for the cells. The advantage of such a selection mechanism is that multi-copy integrants are enriched leading to higher levels of heterologous protein production. Apart from the use of this marker gene in *K. lactis,* Selton and co-workers (2000) also demonstrated its use in *S. cerevisiae*,

E. coli, *Bacillus subtilis* and *Bacillus licheniformis*. The main criterion for the use of this system is the absence of any acetamide activity in the host strain. Another advantage of this marker system is that it can be recycled through a counter selection using fluoroacetamide. Cells expressing acetamidase converts fluoroacetamide to the toxic compound fluoroacetate. If the acetamidase encoding gene is flanked by sequences that allow recombination (e.g. *Lox* cassette) cells can be selected that have lost the marker gene (Selton et al., 2000; van Ooyen et al., 2006). This system is similar to the *S. cerevisiae URA3* counter selection using 5-FOA (5-Fluoroorotic Acid) (Boeke et al., 1987).

Most of the promoters that are available and that have been used successfully for heterologous expression are *S. cerevisiae* promoters. These include constitutive (e.g. *PGK*) as well as regulatable promoters (*GAL1, GAL7, ADH2* and *PHO5).* The most extensively used *K. lactis* promoters are the *KlADH4* and the inducible *LAC4* promoter (Gellissen et al., 1992; van Ooyen et al., 2006). The *KlADH4* is a mitochondrial located alcohol dehydrogenase and strains with impaired sugar fermentation (i.e. Rag^-) need the addition of exogenous ethanol for induction. Furthermore, this gene is not repressed by glucose and is induced in strains that do produce ethanol from fermentable carbon sources. Transcriptional activity can be further increased in these strains with the addition of exogenous ethanol. The advantage of such a promoter in heterologous driven expression is that expression can be regulated with the addition of the inducer, in this case ethanol, independently of the carbon source used (Saliola et al., 1999). Saliola et al. (1999) identified an ethanol-responsive element in the promoter region of *KlADH4*. When this element was inserted into either the native *LAC4* promoter or the *S. cerevisiae PGK* promoter it conferred ethanol-dependent induction of these promoters. The *LAC4* gene encodes a native lactase (β-galactosidase) in *K. lactis*. The *LAC4* promoter (P_{LAC4}) is often used in heterologous expression due to its strength and inducible expression. One negative aspect of this promoter is its functionality in *E. coli* which can be problematic in the assembly of an expression vector in this bacterium especially if the heterologous gene product is deleterious. Colussi and Taron (2005) constructed a P_{LAC4} promoter lacking the sequences on this promoter that resemble the bacterial Pribnow box element (designated $P_{LAC4\text{-}PBI}$). The mutated promoter reduced expression of a reporter protein (GFP) by approximately 87% in *E. coli* and expression of human serum albumin in *K. lactis* were comparable to expression using the wild-type promoter. Furthermore, a number of proteins that are toxic in *E. coli* (including the protease bovine enterokinase and the mouse transthyretin) were cloned using this altered promoter (Colussi and Taron, 2005).

K. lactis can also be used as a host for production of heterologous proteins as such proteins may be readily excreted into the medium. For this, two types of strategies are considered: one is based on the expression of chromosomally integrated foreign protein genes (which are highly stable through mitosis), and the other relies on multi-copy plasmid vectors carrying the foreign gene (maintained at a high gene dosage). Both approaches have given successful examples: prochymosin (*LAC4* integration) (van den Berg et al., 1990), glycoprotein E2 from hepatitis C virus (*TRP1* integration) (Mustilli et al., 1999) and α-galactosidase (rDNA integration)

(Bergkamp et al., 1992) was produced from chromosomally integrated genes. pKD1 plasmid-based vectors were employed to express, for example, human serum albumin (Fleer et al., 1991a), human interleukin-1 beta (Fleer et al., 1991b), cellulose, lipase and polygalacturonase (Müller et al., 1998). Multiple integration of a plant α-galactosidase gene into ribosomal DNA produced a high level of secreted production of this enzyme, 250 mg l^{-1} in comparison to 90 mg l^{-1} when expression was pKD1 based (Bergkamp et al., 1992). Targeted integration is however not as efficient as is the case of *S. cerevisiae* and efficiency of integration often depends on the targeted loci (Zeeman et al., 1998). It was shown recently that targeted integration can be dramatically increased with the deletion of the *KlKu80* (Kooistra et al., 2004). Using the *KlADE2* as targeted loci with varying lengths of homologous sequences, site specific integration efficiency in wild-type *K. lactis* varied from 0% to 80%. In a *KlKu80* deletion mutant, correctly targeted integration efficiency was improved to > 97% independent of the length of the homologous flanking regions. Targeted integration does however have the disadvantage of reduced number of heterologous gene copies in comparison to multicopy vectors. One method to ensure multi-copy integration is through the targeting of the rDNA loci (Bergkamp et al., 1992).

In addition to the above-mentioned heterologous proteins, more that 40 proteins have been produced with *K. lactis*. These proteins derived from bacteria, fungi, viruses, plants, and mammals showing the importance of this yeast in heterologous protein production (reviewed by van Ooyen et al., 2006).

Colussi and Taron (2005) reported on the development of an integration vector (pKLAC1) containing a number of advances discussed. This vector, developed at New England Biolabs, Inc. and DSM Biologics Company B.V., contains the modified *K. lactis* $P_{LAC4\text{-}PBI}$ promoter, DNA encoding the *K. lactis* α-mating factor (KL-α-MF) secretion domain, a multiple cloning site, the *K. lactis LAC4* transcription terminator (TT), and the *Aspergillus nidulans* acetamidase selectable marker gene (amdS) expressed from the *S. cerevisiae ADH2* promoter. An *E. coli* replication origin (ori) and ampicillin resistance gene (ApR) are present for propagation of pKLAC1 in *E. coli*. This vector forms part of the *K. lactis* protein expression kit together with the industrial strain GG799 commercially sold by New England Biolabs Inc. (USA). This expression system can be used for research purposes; however, a license should be obtained from New England Biolabs, Inc. (USA) or DSM Biologics Company B.V. (The Netherlands) for commercial purposes.

18.5 Yarrowia lipolytica

18.5.1 History

Yarrowia lipolytica [(Wickerham, Kutzman and Herman) van der Walt and von Arx] is the only ascosporic member of the genus *Yarrowia* van der Walt and von Arx. *Y. lipolytica* was identified as the teleomorph of *Candida lipolytica* and originally

described as *Endomycopsis lipolytica* then as *Saccharomycopsis lipolytica* and finally as *Yarrowia lipolytica* (van der Walt and von Arx, 1980). *Yarrowia lipolytica* is heterothallic displaying dimorphic growth forming yeast cells, true hyphae and pseudohyphae, depending on growth conditions. *Y. lipolytica* strains are frequently isolated from dairy products and meat products, do not ferment sugars and are not considered as a pathogenic species. This lack of fermentative capabilities allows their easy elimination from for example dairy products (Barth and Gaillardin, 1997; Kurtzman, 1998a).

18.5.2 Introduction

Interest into the potential of *Y. lipolytica* as a heterologous protein producer and secretor aroused from the fact that this yeast naturally secretes several proteins, including proteases, lipases, phosphatases, RNAse and esterase. Yield of alkaline extracellular protease (AEP) secreted under inductive conditions by the wild-type organism ranged between 1–2 g l^{-1}. The good yield and secretion capability, together with the fact that *Y. lipolytica* is considered as non-pathogenic and that several processes based on the yeast were awarded GRAS status by the FDA, made *Y. lipolytica* an excellent candidate for genetic exploration and exploitation as a alternative expression host (Barth and Gaillardin, 1997).

Most of the groundbreaking work in terms of genetic elucidation, manipulation and tools to assist in manipulation of *Y. lipolytica* was and still is being performed at the laboratories directed by Prof. Claude Gaillardin in Grignon, France. Inbreeding programs resulted in genetically tractable lines, allowing complementation and linkage studies to become more feasible. *Y. lipolytica* can be transformed with either replicative vectors (containing centromeric as well as replicative functions) or integrative vectors (integration occurs usually by recombination between the plasmid sequence homologous to a chromosomal target sequence) (Vernis et al., 2001; Gaillardin et al., 1985).

The elucidation of the genomic organization and finally the complete sequence of the genome of an organism are necessary in order to fully utilize the power of molecular biology and molecular manipulation. Studies into the genomic organization of several *Y. lipolytica* strains revealed the presence of six chromosomes and that the overall structure of the genome is conserved between different isolates (Casarégola et al., 1997). Two isolates of *Y. lipolytica* were subjected to genome sequencing driven by the Génolevures projects, resulting in the assembly of the complete genome of 20.5 Mb in size (excluding the rDNA) containing approximately 6700 open reading frames (Casarégola et al., 2000; Dujon et al., 2004). The *Y. lipolytica* genome was found to be the largest of the hemiascomycetous species, but with the lowest gene density due to the amount of non-coding DNA present in the genome. The elucidation and annotation of the genome, opens the possibility for intricate pathway engineering by laying down the blueprint describing each component involved in cellular processes.

Y. lipolytica has two major advantages over the established *S. cerevisiae* system when it comes to the production of human therapeutic proteins. The first is that it allows for predominantly co-translational translocation of newly synthesized proteins into the endoplasmic reticulum (also the case in higher eukaryotic organisms), in contrast to the post-translational translocation predominant in *S. cerevisiae* (Biosramé et al., 1998). The second advantage is that recorded glycosylation of a protein from human origin indicated short oligosaccharide chains (8–10 mannose residues added to the core oligosaccharides), compared to the long oligosaccharide chains (50–150 mannose residues) added by *S. cerevisiae* (Madzak et al., 2005).

Müller and co-workers (1998) performed an evaluation of different yeast expression platforms for the production of heterologous proteins encoded by genes originating from three filamentous fungal strains. Evaluation criteria included transformation efficiency, degree of glycosylation, growth of recombinant host and the amount of active recombinant enzyme produced by the host. *Y. lipolytica* was found to be the most promising host tested, thereby outcompeting *S. cerevisiae*, *Schizosaccharomyces pombe*, *K. lactis* and *H. polymorpha.*

18.5.3 Yarrowia lipolytica Strains

A detailed review on the characteristics and properties of all *Y. lipolytica* strains used for protein expression was published by Madzak and co-workers (2004). This section would only indicate the highlights in the construction of the most widely used strain and its derivatives.

Several *Y. lipolytica* host strains were engineered for the expression of recombinant proteins. Engineering of the strains entailed the identification and subsequent disruption of a wide variety of marker genes and the use of antibiotic resistance markers, allowing manipulation of industrial *Y. lipolytica* strains not harbouring any auxotrophic markers (Madzak et al., 2004). The importance of *Y. lipolytica* as a secretor of heterologous proteins was advanced by the construction of the W29 wild-type derived Po1d strain (le Dall et al., 1994). The strain contained an alkaline extracellular protease (AEP) deletion, which was necessary since AEP was produced in abundance and could cause havoc by degrading secreted recombinant proteins. This strain also shared the same high secretion capability present in W29 wild-type and its derivatives. It also contained an integrated copy of the *SUC2* gene from *S. cerevisiae*, facilitating sucrose utilization as an alternative carbon source and allowing efficient and cost-effective growth of the recombinant strain on molasses. Further improvements to the strains in terms of heterologous protein production included deletion of the acidic extracellular protease, effectively eliminating the major proteases secreted into the culture medium. Another derivative (Po1g) was fitted with a pBR322 docking platform to allow easy integration of pBR322 derived expression constructs (Madzak et al., 2000; Madzak, 2003).

18.5.4 Genetics and Molecular biology

Functional gene expression in *Y. lipolytica* is derived from the expression cassette, including a promoter, the recombinant gene to be produced (with or without a signal peptide) and a terminator region recognized by the transcription machinery of *Y. lipolytica*. The expression cassette is also linked to a marker gene (either auxotrophy complementation or antibiotic resistance) that allows selection of transformants carrying the expression cassette and flanked by either a target region that allows recombination of the expression cassette with homologous regions in the genome (for integrative vectors) or by regions allowing replicative and centromeric functions (for episomal maintenance of the plasmid). Most of the expression construct attention was focused on integrative vectors instead of episomal vectors, due to the limited copy number of the episomal vectors (1–3 copies/cell), together with the requirement for continual selective pressure and limited gene expression (Madzak et al., 2000).

Transformation of competent *Y. lipolytica* with linearized integrative expression cassettes based on a single crossover recombination event (using the lithium acetate method) resulted in transformation efficiencies of up to 10^6 transformants μg^{-1} DNA where more than 80% of the transformants will harbor a single copy of the expression cassette integrated at the correct site into the genome (Xuan et al., 1988; Barth and Gaillardin, 1996). The integrative vectors were further optimized in such a way that the transformation construct that would integrate into the genome would be free of any bacterial DNA (Pignéde et al., 2000). These 'auto-cloning' vectors consist of a yeast cassette, flanked by a ≥8 bp restriction site, separating the bacterial part from the yeast cassette. Removal of the bacterial DNA is achieved by digesting the circular plasmid with the appropriate enzyme, separation of the two moieties using agarose gel electrophoresis, isolation of the yeast cassette for transformation of the host to yield a transformant devoid of bacterial DNA and resistance markers that could complicate commercial and industrial applications. A typical example of such an auto-cloning integrative expression construct used in *Y. lipolytica*, together with the most common elements used in the construct, is graphically illustrated in Fig. 18.2.

Le Dall and co-workers (1994) set out to increase expression cassette copy number by creating different sets of vectors targeted to the conserved and highly abundant rDNA regions in the genome of *Y. lipolytica*. These vectors contained sequentially truncated promoter regions of the *URA3* marker gene, thereby effectively preventing growth of the transformants when a single copy of the marker gene is present under selective conditions. Up to 60 copies of the expression cassette integrated into the genome when the *ura3d4* defective allele (containing only 6 bp or the original promoter upstream from the initiator ATG codon) was used as a marker gene for selection of positive transformants - albeit with a very low transformation efficiency of only 10 transformants μg^{-1} DNA. Integration into the genome occurred mostly as tandem repeats of the cassette into two different chromosomes, with some dispersed copies also observed. Induction of the reporter gene, AEP, however resulted in a decrease and stabilization in copy number of ~10 copies/genome. A good correlation between copy number and AEP production was

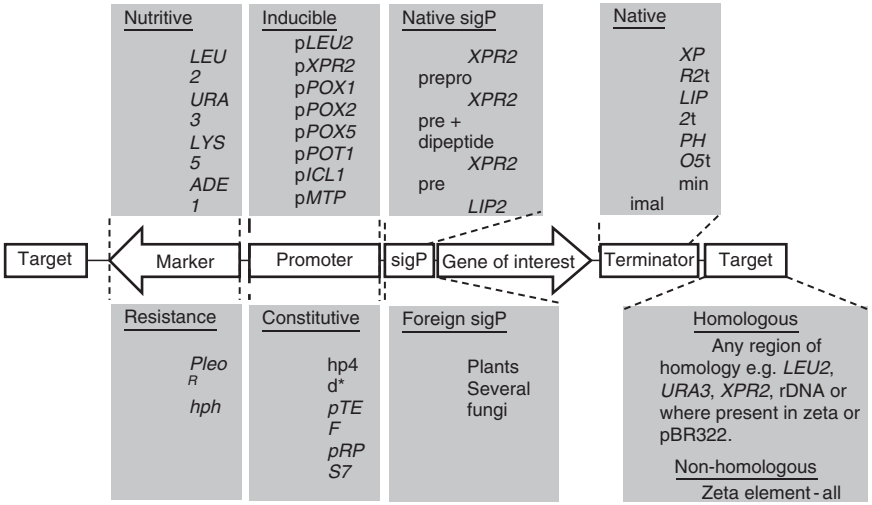

Fig. 18.2 Graphical representation of a typical auto-cloning integrative expression construct used for transformation of *Y. lipolytica*. All elements listed have been reviewed and described in detail by Madzak and co-workers (2004)

observed for up to 10 copies/genome, without causing expression cassette instability and affecting strain viability. This was confirmed in a study performed by Juretzek and co-workers (2001) where results indicated that multi-copy integration occurred for all the target regions predominantly as tandem repeats, with stable copy number of 10–13 copies/genome after induction. It was also observed that transformants exhibiting lower copy number (3–6 copies/genome) showed an increase to the level of 10–13 copies/genome after successive cultivation on selective media. The conclusion of the study indicated that the determining factor for multi-copy transformants seems to be the requirement for multiple copies of the defective marker to restore the ura− phenotype, and not the presence of multiple target sites for integration.

The use of a PCR-mediated gene deletion/disruption method employing the counter selectable *URA3* marker gene for use in *Y. lipolytica* has been described by Nicaud and co-workers (1998). The method relies on constructing a promoter-*URA3*-terminator for gene replacement, verification of deletion and use of a promoter-terminator cassette in a second round of transformation, followed by selection of ura− transformants on 5-fluoroorotic acid (5-FOA). The presence of a functional intact *URA3* in the genome will allow the cells to metabolize the 5-FOA where the metabolite becomes toxic to the cells, thereby allowing only cells without the functional intact *URA3* to grow. The *URA3* can then be re-used for the next gene deletion (Boeke et al., 1987). This method has been successfully employed in *Y. lipolytica* for the functional analysis of acyl-coA oxidase family as well as functional analysis of some genes involved in the glycosylation pathway (Wang et al., 1999; Barney-Verdier et al., 2004). The major disadvantages of this method are that it is limited to the *URA3* marker and that it is laborious and time consuming.

With the publication of the first series of sequences from the Génolevures project, an efficient system was developed for the rapid gene disruption and marker recycling in *Y. lipolytica* (Fickers et al., 2003). The system is based, like the 5-FOA system, on a promoter-marker-terminator cassette to facilitate in the gene disruption, but in this method the marker is flanked by *lox* sequences. The *lox* sequences serve as template for heterologous expressed *Cre* recombinase (transcribed from an episomal plasmid of which the recipient strain can be cured of), which will, with high efficiency, recombine the two *lox* sites, effectively looping out the marker gene. Any marker gene can be used with this system, making multiple gene disruptions, followed by a once off marker rescue, possible. The use of the antibiotic resistance marker gene *hph* allows for the manipulation and engineering of all *Y. lipolytica* strains, especially industrial strains that often lack the conventional auxotrophic marker. The only disadvantage of the method is that every recombination event of the *lox* sites, leaves a recombined *lox* scar in the genome. Multiple deletions would result in several *lox* sites, which could theoretically be recombined when *Cre* recombinase is expressed in the system, allowing for the possibility of looping out of stretches of non-essential DNA that could affect the experimental approach.

Buckholz and Gleeson (1991) reviewed yeast systems for the commercial production of heterologous proteins and indicated that a mere five recombinant heterologous proteins were reportedly produced using *Y. lipolytica* as an expression hosts. A recent comprehensive review on *Y. lipolytica* as a heterologous expression host listed 42 heterologous proteins produced in the same system, with this number continually increasing (Madzak et al., 2004). These protein encoding genes originated from viruses, eubacteria, cyanobacteria, fungi, plants and mammals, covering proteins with molecular weights of 10 kDa–116 kDa in size. The secretion machinery of *Y. lipolytica* also recognized several foreign secretion signals, processed them and secreted functional protein into the extracellular environment. The production of laccase from *Pycnoporus cinnabarinus* and α-foetoprotein from *Homo sapiens* was roughly three- and two-fold, respectively, higher in *Y. lipolytica* when compared to *P. pastoris. Y. lipolytica* also outperformed *K. lactis* during the production of anti-Ras scFv.

Recombinant protein yields in *Y. lipolytica* have been moderate with the maximum yield of 1 g l^{-1} obtained for the *E. coli* derived amylolytic protein. The low production efficiencies of recombinant human proteins in this system could be explained by the difference in codon usage by the host, since these heterologous genes have not been codon optimized for *Y. lipolytica* (Madzak et al., 2004). A recent paper indicated the overproduction *LIP2* from *Y. lipolytica* in up to 3 g l^{-1} quantities using a *Y. lipolytica* strain with a multi-copy expression cassette (Aloulou et al., 2007). It should however be mentioned that at present, no industrial processes for the production of recombinant proteins by *Y. lipolytica* exist (Gellissen et al., 2005).

Y. lipolytica has been extensively researched and adapted as a host for the production of heterologous recombinant proteins from a wide variety of sources. Strains have been optimized for secretion, to accommodate several vector systems and allow stable integration into the genome of the organism. Tools to perform

yeast system engineering on DNA level have also been developed, to allow stable and rapid deletion, disruption and replacement of targets in the genome. The completion of the *Y. lipolytica* genome sequence will facilitate studies into pathway engineering, factors effecting high level expression of recombinant proteins, and assist in the identification of new production friendly promoters. Recent work done by Song and co-workers (2007) solved the first step in the production of therapeutic glycoproteins containing N-linked human-compatible sugars in *Y. lipolytica* by deleting the *Y. lipolytica OCH1* gene, resulting in a strain that can be used to produce glycoproteins lacking the outer chain mannoses.

Y. lipolytica as an expression system has also made its debut as being commercially available through a biotechnology company. This system, known as the YLEX Expression kit, has been commercialized by researchers at INRA (France) in collaboration with Yeastern Biotech Co. located in Taiwan (http: //www.yeastern.com) (C. Madzak, personal communication). The system offers a yeast strain (Po1g), two vectors (for intra- and extracellular expression), two primers (for insert sequencing) and a one-step transformation kit. This commercialized product should lead to the increased use of *Y. lipolytica* as an alternative expression host.

All the aspects in terms of *Y. lipolytica* as a heterologous producer of recombinant proteins have been optimized in order to make *Y. lipolytica* a definite candidate for the expression of any given protein than needs to be produced.

18.6 Schwanniomyces occidentalis

Schwanniomyces occidentalis (formerly known as *Schwanniomyces castellii*) is an amylolytic species that belongs to the same subfamily (*Saccharomycoideae*) as the genera *Saccharomyces*, *Kluyveromyces*, *Pichia* and *Hansenula*. *Sw. occidentalis* is important because of its ability to degrade starch completely. Starch is degraded by two secreted amylases, an α-amylase (encoded by thy *AMY1* gene) and a glucoamylase (encoded by the *GAM1* gene) (Boze et al., 1989). The production of enzymes is induced in the absence of glucose by the presence of maltose or starch. The ability of this species to grow in inexpensive media makes it a useful organism for production of heterologous proteins (Spencer et al., 2002). In addition, *Sw. occidentalis* is a potentially important host for large-scale commercial production of heterologous gene products because it can secrete proteins greater than 140 kDa efficiently into the culture medium (Oteng-Gyang et al., 1981; Wilson and Ingledew, 1982; Sills et al., 1984; Deibel et al., 1988), does not hyperglycosylate secreted proteins, and does not secrete measurable quantities of proteases (Deibel et al., 1988).

Klein and Favreau (1988) developed an efficient transformation and cloning system for *Sw. occidentalis* based on *ade2* mutants and the *ADE2* gene from *Sw. occidentalis* as a selectable marker. This system used a modification of the spheroplast procedure described by Beggs (1978). In the transformants derived from the *ade2* mutant host, the heterologous DNA was either integrated into the

chromosome or maintained as an extrachromosomal element without detectable mitotic loss. The episomal DNA was found to be present in a variety of plasmids of different molecular mass as a result of a high level of rearrangements. Pointek et al. (1998) designed expression vectors based on the *Sw. occidentalis*-derived autonomously replicating sequence (*SwARS*) and the *S. cerevisiae*-derived *TRP5* sequence for plasmid propagation and selection in yeast hosts, an origin of replication and an ampicillin-resistance sequence for propagation and selection in a bacterial host. These vectors share components for selection and propagation suitable for *S. cerevisiae*, *Sw. occidentalis* and *P. stipitis* (Dohmen et al., 1989) and the basic design of these vectors provides the potential to assess gene expression in a wide range of tryptophan-auxotrophic yeasts. In contrast to the results obtained by Klein and Favreau (1988, 1991) regarding heterologous DNA integration, the plasmids carrying the *S. cerevisiae*-derived *TRP5* gene and *SwARS* sequences do not recombine and yield an average of 5–10 copies per cell under selective conditions in the *Sw. occidentalis* host (Dohmen et al., 1989). Vector systems for *Sw. occidentalis* have been investigated and developed to some extent (Klein and Favreau, 1991), but no *CEN* (centromere) plasmids are available at the moment.

18.7 Methylotrophic Yeasts

Methylotrophic yeasts have gained increasing interest for fundamental research and as attractive hosts for the production of biologically active proteins (Hollenberg and Gellissen, 1997). These yeasts comprise a group of microorganisms able to use methanol as carbon source and energy. Adaptation to growth on methanol is associated with induction of methanol oxidase (also referred to as alcohol oxidase), dihydroxyacetone synthase and several other enzymes involved in methanol metabolism. The most spectacular increase, however, is seen with alcohol oxidase, which is virtually absent in glucose-grown cells, but can account for over 30% of the cell protein in methanol-grown cells. Extensive proliferation of peroxisomes, accounting for over 80% of the cell volume, is also observed in methanol-grown cells (Veenhuis et al., 1983). Due to these characteristics, methylotrophic yeasts have gained the attention of biochemists, molecular biologists, cell biologists, biotechnologists, microbiologists and chemists in academics and industry.

The use of these organisms in fundamental research is mainly related to studies of peroxisome homeostasis and nitrate assimilation (van der Klei and Veenhuis, 1996; Perez et al., 1997; Veenhuis et al., 2000). The peroxisomes contain the key enzymes involved in methanol metabolism, namely alcohol oxidase (AOX), dihydroxyacetone synthase (DHAS) and catalase (CAT). The two proteins AOX and DHAS may constitute over 60% of total cellular protein under these conditions. This illustrates that the genes encoding these proteins are controlled by very strong promoters. This feature contributed to recognizing the methylotrophs as attractive hosts for the production of heterologous proteins.

18.8 Hansenula polymorpha (Pichia angusta)

18.8.1 History

Hansenula polymorpha is one of the species of *Hansenula* that were transferred to the genus *Pichia* based on the formation of hat-shaped ascospores (Kurtzman, 1984). This lead to the proposed name change from *H. polymorpha* to *Pichia angusta* [(Teunisson, Hall and Wickerham) (Kurtzman, 1984)]. With the move to the *Pichia* genus a nomenclatural difficulty arose. The name *Pichia polymorpha* was previously used for the species currently known as *Debaryomyces polymorpha*. Due to the unavailability of the name *polymorpha, Pichia angusta* was selected because *Hansenula angusta* is an obligate synonym of *H. polymorpha* (Kurtzman, 1998b). However, most researchers prefer the original name of *H. polymorpha* and although taxonomically incorrect we will for the sake of simplicity refer to this yeast as *H. polymorpha*.

18.8.2 Introduction

H. polymorpha has some specific advantages over other methylotrophic yeasts (such as *P. pastoris* and *C. boidinii*), being more thermotolerant and capable to grow at higher rates on simple, defined media. The relatively high optimal growth temperature for *H. polymorpha* (37°C–43°C) may be favorable for the production of mammalian proteins and furthermore has the advantage that it allows for better management and reduces the risk of contaminations in large scale fermentations (Gellissen, 2000).

Extensive proliferation of peroxisomes, accounting for over 80% of the cell volume, is observed in methanol-grown cells (Veenhuis et al., 1983). A characteristic feature of peroxisomes is that they are inducible. In *H. polymorpha*, peroxisomes can be induced by methanol, ethanol, primary amines, D-amino acids, L-α hydroxyl acids and purines (Veenhuis and Harder, 1988). The advantage of the accumulation of proteins in peroxisomes is obvious in cases when expressed proteins are toxic for the host organism, where the peroxisomal membrane forms a barrier, and thereby preventing that the proteins can exert their toxic activity to the yeast cell. Another potential advantage of storage in peroxisomes is the absence of modifying enzymes which prevent undesired modifications such as glycosylation. In addition, proteins which are sensitive to proteolytic degradation are protected from proteolysis inside the matrix of the peroxisome.

H. polymorpha also proved an excellent model by which to study the nitrate assimilation pathway and its transcriptional regulation (Brito et al., 1999). All these facts have promoted important development of the genetic analysis and molecular biology tools for *H. polymorpha* (Rezaee, 2003).

18.8.3 Genetics and Molecular Biology

An essential tool for the construction of recombinant *H. polymorpha* strains are *E. coli–H. polymorpha* shuttle vectors. An important feature of these vectors is the selectable marker genes, which can functionally complement various auxotrophic *H. polymorpha* strains. Commonly used marker genes are *H. polymorpha LEU2, URA3, TRP3* and *ADE11*, the *S. cerevisiae* genes *LEU2, URA3* as well as *C. albicans LEU2* (Roggenkamp et al., 1986; Merckelbach et al., 1993; Agaphonov et al., 1994; Bogdanova et al., 1995). Unlike *S. cerevisiae, H. polymorpha* does not harbour any natural plasmids, but with the isolation of auxotrophic mutants, like ura− strains (Roggenkamp et al., 1986) and leu− strains (Gleeson et al., 1986), and complementation by the respective genes form *S. cerevisiae*, introduction of plasmid DNA could be established. In addition to auxotrophic markers a number of alternative selection systems have been used successfully including G418 (Liu et al., 2005), phleomycin (Zurek et al., 1996) and zeocin (Song et al., 2003).

Production systems based on *H. polymorpha* rely on the use of various promoter elements, both inducible and constitutive. Commonly used promoter elements are those derived from genes of the methanol metabolism pathway, which are strongly inducible. The methanol oxidase promoter, dihydroxyacetone synthase promoter and the promoter of formate dehydrogenase gene are fully repressed by excess glucose and are strongly induced by methanol. With the employment of the abovementioned promoters, various attractive induction strategies can be designed. For instance, in the case of harmful recombinant proteins, first biomass can be generated, followed by an alteration in composition of the growth medium, thereby inducing the expression of the heterologous gene (Rezaee, 2003). Derepression of these promoters is also possible under glucose- or glycerol-limiting conditions (e.g. in carbon-limited chemostat cultures). Other inducible promoter elements that have been identified in *H. polymorpha* include elements derived from genes of the nitrate metabolism, *YNT1, YNR1* and *YNL1* (Avila et al., 1996; Brito et al., 1996; Perez et al., 1997) and the repressible acid phosphatase (*PHO1*) promoter (Phongdara et al., 1998). These promoters could be used as new control elements for protein production in *H. polymorpha*. Promoters for constitutively expressed gene products have also been identified in *H. polymorpha*, such as the promoter of the plasma membrane H^+-ATPase (P*PMA1*) (Cox et al., 2000) and the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*PGAP*) (Heo et al., 2003).

Efficient and reliable transformation procedures for *H. polymorpha* have been developed. Yeast cells can be transformed using whole-cell methods according to the lithium acetate-dimethylsulfoxide method described by Hill et al. (1991), by adding PEG (Dohmen et al., 1991) or by electroporation (Faber et al., 1994). In *H. polymorpha*, a high frequency of plasmid integration is observed despite the presence of an *ARS* (autonomously replicating sequence) (Sohn et al., 1996) on a vector. Transformation results in mitotically stable strains containing different copies of an integrated expression cassette exhibiting a head-to-tail arrangement (Gellissen and Hollenberg, 1997). They are integrated by non-homologous recombination and strains with up to 100 copies have been identified (Janowicz et al.,

1991; Gellissen et al., 1994; Gatzke et al., 1995). Targeted integration is achieved through recombination following transformation of linear plasmids containing homologous sequences which are targeted to specific loci in the genome. A variety of target sequences have been used successfully including the *LEU2* gene and *HARS36* (Agaphonov et al., 1999) or the *MOX/TRP* locus (Agaphonov et al., 1995; Machin et al., 2001; Song et al., 2003).

Recently a number of researchers have shown that integration at the rDNA loci is very good alternative (Cox et al., 2000; Klabunde et al., 2002, 2003, 2005; Liu et al., 2005). The advantage of integration into the rDNA loci includes the use of such a vector for the transformation of variety of yeast species. Klabunde et al. (2003) designed a vector (pTHpH181Hp) with the aim of transforming *H. polymorpha*, *S. cerevisiae*, *P. stipitis* and *A. adeninivorans*. Apart from a bacterial moiety the vector contained the *TEF1* promoter from *A. adeninivorans* linked to the *hph* gene from *E. coli* followed by the *PHO5* terminator from *S. cerevisiae.* This region, obtained from the pAL-HPH1 plasmid (Rösel and Kunze, 1998) served as selection against hygromycin in the different yeast species. Different regions of the *H. polymorpha* rDNA unit were tested and it was shown that the inclusion of the putative promoter region of the 35S rDNA precursor together with the ETS region and the full-length 18S rDNA sequence allowed successful transformation of all the yeast species tested. In addition to the 18S rDNA region, the 25S region was also shown to allow integration of homologous rDNA containing plasmids using the *S. cerevisiae* derived *URA3* gene as selectable marker (Klabunde et al., 2002) or the geneticin (G418) as resistance gene (Liu et al., 2005). No difference was seen in terms of expression of a firefly luciferase (Luc) reporter gene when transformed *H. polymorpha* was tested using either an 18S or 25S containing vector (Liu et al., 2005).

H. polymorpha is currently used as an expression system by Rhein Biotech GmbH (Germany) to produce a recombinant hepatitis B vaccine which is sold under the name Hepavax-Gene®. This company uses the *Hansenula* expression system for production of recombinant proteins, and also licenses the technology to other companies. The complete genomic sequence of *H. polymorpha* was determined by Rhein Biotech who commissioned Qiagen Genomic Services (Qiagen, GmbH, Germany). The *H. polymorpha* genome consists of approximately 9.5 Mb organized as six chromosomes ranging in size from 0.9 to 2.2 Mb containing approximately 5933 open reading frames (Ramezani-Rad et al., 2003). Currently the genome data is not in the public domain and access to the genome data can be obtained following a material transfer agreement. Part of the *H. polymorpha* genome sequence was also determined through the Génolevures project. In this instance coverage of 0.5 genome equivalents was completed and approximately 2500 novel protein-coding genes were identified (Blandin et al., 2000).

The need to establish a reliable system for the production of heterologous membrane proteins is emphasized by the observation that many human diseases are caused by the malfunctioning of membrane proteins, therefore, such a system would be of the utmost importance for various reasons, such as the facilitation of rational drug design. In addition, fundamental research such as resolving the structure and functionality of important membrane proteins rely on the availability of

relative large amounts of biologically active membrane proteins. In *H. polymorpha*, excessive peroxisomal membranes are easily developed that hardly contain protein components and thus are an excellent storage place for heterologous membrane proteins (Veenhuis et al., 1990). Localization to these membranes can be mediated by the targeting signal of the *H. polymorpha* peroxisomal membrane protein Pex3p (Baerends et al., 1996) which suggests that *H. polymorpha* could be a significant tool in the production of membrane proteins.

18.9 Pichia pastoris

18.9.1 History

Pichia pastoris [(Guilliermond) Phaff] is a methylotrophic yeast that can grow on methanol as sole carbon and energy source. The Phillips Petroleum Company was the first to develop media and protocols for cultivating *P. pastoris* on methanol in continuous cultures. An efficient ultra-high cell density (>130 g dry cell weight per liter) (Cereghino and Gregg, 2000) fermentation process with high biomass productivity (>10 g l^{-1} h^{-1}) was developed for this yeast (Sreekrishna and Kropp, 1996). During the 1970s, *P. pastoris* was considered as a potential source of single-cell protein for feedstock due to its ability to utilize methanol as sole carbon source. However, the oil crisis of the 1970s caused a dramatic increase in the cost of methane (the source of the methanol) and the economics of this process, while impressive from a fermentation standpoint (approximately $5 per pound of protein), was clearly an order of magnitude higher in comparison to the cost of a pound of soybeans, which was the major alternative source of animal feed. This resulted in Phillips Petroleum Company investing its efforts in developing this yeast as an expression system for the production of recombinant proteins proving to be a worthwhile endeavor (Sreekrishna and Kropp, 1996). *P. pastoris* has gained widespread attention as an expression system because of its ability to express high levels of heterologous proteins. As a result, recombinant vector construction, methods for transformation, selectable marker generation and fermentation methods have been developed to exploit the potential of this system (Rosenfeld, 1999). Research Corporation Technologies (Tucson, Arizona, USA) are the current holders of the patent for the *P. pastoris* expression system and Invitrogen Corporation (Carlsbad, California, USA), has an exclusive license to sell the *Pichia* Expression Kit to scientist for academic research purposes (Macauley-Patrick et al., 2005; www.invitrogen.com).

18.9.2 Introduction

P. pastoris is a homothallic, ascomycetous yeast that can be manipulated by classical genetic methods (Cregg and Madden, 1988; Cregg et al., 1998). The conceptual basis for the *P. pastoris* expression system stems from the observation that some of the

enzymes required for methanol metabolism are present at substantial levels only when cells are grown on methanol (Egli et al., 1980; Veenhuis et al., 1983). Unlike homothallic strains of *S. cerevisiae*, which are diploid, *P. pastoris* remains haploid unless forced to mate. Strains with complementary markers can be mated by subjecting them to a nitrogen-limited medium. After one day on this medium, cells are shifted to a standard minimal medium supplemented with nutrients designed to select for complementing diploid cells (not self-mated or non-mated parental cells). The resulting diploids are stable as long as they are not subjected to nutritional stress. To obtain spore products, diploids are returned to the nitrogen-limited medium, which stimulates them to proceed through meiosis and sporulation. Spore products are handled by random spore techniques rather than micromanipulation, since *P. pastoris* asci are small and difficult to dissect. Yet most standard classical genetic manipulations, including mutant isolation, complementation analysis, backcrossing, strain construction and spore analysis, can be accomplished (Cereghino and Gregg, 2000).

18.9.3 Genetics and Molecular Biology

Techniques required for the genetic manipulation of *P. pastoris*, such as DNA-mediated transformation, gene targeting, gene replacement and cloning by functional complementation, are similar to those described for *S. cerevisiae*. *P. pastoris* can be transformed by electroporation (Becker and Guarente, 1992), a spheroplast generation method (Cregg et al., 1985; Sreekrishna et al., 1987), or whole cell methods such as those involving lithium chloride (Ito et al., 1983) and polyethylene glycol (Dohmen et al., 1991). The introduced DNA can establish itself in two ways: integration into chromosomal DNA by homologous recombination or autonomous replication as a circular plasmid. As in *S. cerevisiae*, *P. pastoris* exhibits a tendency for homologous recombination between genomic and artificially introduced DNA's. Cleavage of a *P. pastoris* vector within a sequence shared by the host genome stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus (Cregg and Madden, 1987). Gene replacements occur at lower frequencies than those observed in *S. cerevisiae* and appear to require longer terminal flanking sequences to efficiently direct integration (Cregg and Russel, 1998).

The majority of heterologous protein production in *P. pastoris* is based on the fact that enzymes required for the metabolism of methanol are only present when cells are grown in methanol (Egli et al., 1980). Therefore, although other promoter options are available for the production of foreign proteins in *P. pastoris* (extensively reviewed by Cereghino and Gregg (2000)), the *AOX* promoters have been the most widely used (Cereghino et al., 2001). However, there are circumstances in which the *AOX* promoter may not be suitable. Promoters that are not induced by methanol are preferred for the production of food related products since the petroleum related compound methane, is a source of methanol. A second consideration is that methanol is a potential fire hazard, especially with the large quantities that

are needed for large-scale fermentations (Cereghino and Gregg, 2000). Alternative promoters to the *AOX1* promoter include the *P. pastoris GAP* (glyceraldehyde 3-phosphate dehydrogenase), *FLD1* (glutathione-dependent formaldehyde dehydrogenase), *PEX8* (a peroxisomal matrix protein), *YPT1* (a GTPase involved in secretion) and more recently, *ICL1* (isocitrate lyase) promoters (Cereghino and Gregg, 2000; Macauley-Patrick et al., 2005).

Few selectable marker genes have been described for *P. pastoris.* Initially, markers were limited to biosynthetic pathway genes including *HIS4* from either *P. pastoris* or *S. cerevisiae*, *ARG4* from *S. cerevisiae* and the *Shble* gene from *Streptoalloteichus hindustanus* which confers resistance to the bleomycin-related drug zeocin (Cregg et al., 1985, 1989; Higgins et al., 1998). An additional set of biosynthetic markers that includes the *P. pastoris ADE1*, *ARG4*, and *URA3* genes have also been described. Each of these selectable markers have been incorporated into expression vectors and a series of host stains containing all possible combinations of *ade1, arg4, his4* and *ura3* auxotrophies has been generated (Cereghino and Gregg, 2000; Cereghino et al., 2001).

The commercial *P. pastoris* Expression Systems supplied by Invitrogen Corporation have a number of expression vectors available. Currently three types of expression kits are available that includes *P. pastoris* strains, expression vectors, transformation reagents, sequencing primers and media. The vectors contain either the inducible *AOX1* or the constitutive *GAP* promoters and the *HIS4* gene for selection. Expressed proteins can be directed to the extracellular environment with either the *S. cerevisiae* α-factor or the native *P. pastoris* acid phosphatase signal sequence (*PHO1*) or alternatively expressed intracellularly.

The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins (for an extensive list of heterologous proteins expressed successfully in *P. pastoris*, refer to the review by Cereghino and Gregg (2000) or visit the website: http: //faculty.kgi.edu/cregg/index.htm, for an updated version of the list). It is noteworthy to indicate that *P. pastoris* can secrete a large variety of proteins, with yields of up to 14.8 g l^{-1} as reported by Werten and co-workers (1999).

A number of proteins synthesized in *P. pastoris* are being tested for use as human pharmaceuticals in clinical trials. However, a major limitation of *P. pastoris* and other yeast expression systems in the production of therapeutic proteins is that they were unable to replicate the exact post-translational modifications of the proteins as they passed through the protein processing pathway (Cereghino and Gregg, 2000). One of these modifications, correct glycosylation of the therapeutic proteins, was found to be critical in therapeutic efficacy of the protein, since non-human glycosylation can reduce the half-life of the protein and eliciting an immunogenic response to the foreign carbohydrate moiety (Helenius and Aebi, 2001). Researchers at Dartmouth College (Hannover, NH, USA) and GlycoFi Inc. (Lebanon, NH, USA) succeeded in manipulating the glycosylation pathway of *P. pastoris* to secrete human glycoproteins with uniform complex *N*-glycosylation patterns (Hamilton et al., 2003). In order to humanize the *P. pastoris* glycosylation pathway, the endogenous yeast glycosylation pathways had to be eliminated together with the introduction of five eukaryotic proteins involved in glycosylation. Correct localization of these

proteins in the glycosylation pathway was critical and involved complex combinatorial approaches between signal sequences and catalytic domains in order to correctly localize active enzymes in the pathway (reviewed by Wildt and Gerngross (2005)). Li and co-workers (2006) used several of these engineered *P. pastoris* strains to study the effect of glycosylation on monoclonal antibodies and observed improved results when the yeast derived antibodies were compared to commercial, mammalian cell-line derived antibodies. This was followed by advancing the system even further to allow the production of terminally sialylated glycoproteins (Hamilton et al., 2006). The construction of the strain allowing terminal sialylation of the complex human *N*-glycosylated proteins, included the deletion of four yeast genes involved in the yeast specific glycosylation pathway followed by the introduction, expression and correct localization of 14 heterologous genes – effectively mimicking the sequential steps of human glycosylation. GlycoFi Inc. was recently acquired by Merck and Co. Inc. shortly after announcing the potential of yeast derived uniform glycosylation to improve expression of therapeutic proteins.

The yeast *P. pastoris* is a useful system for the expression of milligram to several gram quantities of proteins for both basic laboratory research and industrial manufacture. This methylotrophic yeast is particularly suited to foreign protein expression for reasons such as ease of genetic manipulation encompassed by gene targeting, high-frequency DNA transformation, cloning by functional complementation, high levels of protein expression, both intra- and extracellularly, and the ability to perform higher eukaryotic protein modifications, such as glycosylation, disulfide bond formation and proteolytic processing (Cregg et al., 2000). Integrated Genomics completed the sequence coverage of the 9.4 Mb *P. pastoris* genome (although publicly still unavailable), containing approximately 6000 ORF sequences, thereby allowing fast tracking any future developments of the *P. pastoris* expression system due to genomic data availability (http://www.integratedgenomics.com/pichia.html). Simple purification of secreted recombinant proteins is possible due to the relatively low levels of native secreted proteins (Cregg et al., 1993). The humanization of the *N*-glycosylation pathway in *P. pastoris*, the powerful genetic tools, together with its economy of use, make *P. pastoris* the system of choice for heterologous protein expression of industrial as well as biopharmaceutical importance.

18.10 *Pichia stipitis*

The respiratory yeast *P. stipitis* does not produce ethanol during aerobic cultivation (Skoog et al., 1992; Passoth et al., 1996) suggesting that this yeast is suitable for heterologous protein production since it can effectively convert a carbon source to cell mass. More importantly, this yeast can use xylose, the major hemicellulose component in plant cell walls (and the second most abundant renewable carbon source in nature) as sole energy and carbon source. Xylose has to be isomerized to xylulose in a two-step reaction by a NADP(H)-linked reduction to xylitol (catalyzed by xylose reductase encoded by the *XYL1* gene), followed by the NAD^+-linked

oxidation to xylulose (catalysed by the xylulose dehydrogenase encoded by the *XYL2* gene) (Barnett, 1976). Both these genes have been cloned and characterized (Kötter et al., 1990; Amore et al., 1991).

The main emphasis of heterologous expression in *P. stipitis* is based on the improvement of hemicellulosic sugar utilization. Successful expression and/or improved xylose utilization were obtained with expression of the *Cryptococcus albidus* endo1,4-β-xylanase (Morosoli et al., 1993; Passoth and Hahn-Hägerdal, 2000; Görgens et al., 2005), the *Trichoderma reesei* β-xylanase II (*xyn2*) gene (den Haan and van Zyl, 2001) the *Trichoderma reesei* xyn2 and the *Aspergillus kawachii* xynC (both encoding a β-1,4-endoxylanase) and the *Aspergillus niger* xlnD (encoding a β-xylosidase) genes (den Haan and van Zyl, 2003).

Pointek and co-workers (1998) developed a expression system for *P. stipitis* to test different promoter elements using either the native inducible xylose reductase encoding *XYL1* promoter or the *S. cerevisiae* derived *PDC1* and *ADH1* promoter elements together with the *Schwanniomyces occidentalis GAM1* (encoding a glucoamylase) terminator. Propagation and selection was accomplished through the *Sw. occidentalis* autonomous replication sequence (SwARS) and the *S. cerevisiae* tryptophan syntase (*TRP5*) gene and the cellulose encoding *celD* gene from *Clostridium thermocellum* was used as reporter gene. Enzymatic determination of the reporter gene showed that the native *XYL1* promoter provided the highest productivity. Furthermore, the vector system share components for selection and propagation in *P. stipitis, Sw. occidentalis* and *S. cerevisiae* which can potentially be used to assess heterologous expression in these yeast and potentially in other tryptophan auxotrophic yeasts.

Den Haan and van Zyl (2001) evaluated the *P. stipitis XYLI and TKL* (transketolase gene) promoters as well as the *S. cerevisiae PGK1* (phosphoglycerate kinase) promoter using the *T. reesei xyn2* gene as reporter. In addition to these components the vectors also contained the *P. stipitis ARS2* and *URA3* for propagation and selection. In this study it was shown that the *XYL1* promoter is inducible in the presence of xylose and the *TKL* promoter to be constitutive in the presence of either xylose or glucose. The *S. cerevisiae PGK1* promoter was shown to be non-functional in *P. stipitis*. More important, the recombinant β-xylanase enzyme corresponds to the size of the native *T. reesei* enzyme indicating no or very little glycosylation. The availability of a gene expression system utilizing different promoters together with the indication that *P. stipitis* have a low level of glycosylation makes this yeast a good alternative as an expression host. Furthermore, the 12 Mb genome sequence of *P. stipitis* strain CBS 6054 (containing 5841 ORFs) was recently completed by the DOE Joint Genome Institute (JGI) (http: //genome.jgi-psf.org/) making this yeast even more genetically accessible (Jeffries et al., 2007).

18.11 Conclusions

Since no single yeast-based expression platform exists which is optimal for every protein, a suitable host has to be defined for each heterologous gene to be expressed. It would however be ideal to assess several selected organisms as expression platforms

in parallel for optimal product characteristics. The wide-range integrative yeast expression vector systems based on *A. adeninivorans*- and *H. polymorpha*-derived elements (Ilgen et al., 2005; Klabunde et al., 2003, 2005; Terentiev et al., 2004) fulfill the criteria expected of a vector system that could be targeted to the various test species. These criteria include a targeting sequence (such as the conserved NTS2-ETS-18SrDNA-ITS1 region from *H. polymorpha* or the 25S rDNA region from *A. adeninivorans*), a promoter element for expression control of reporter sequence (e.g. the *A. adeninivorans*-derived *TEF1* promoter) and a selection marker that function in all selected organisms (*E. coli*-derived *hph* gene conferring hygromycin B resistance). Heterologous gene expression from these expression platforms was assessed with green fluorescent protein (GFP), phytase from *Aspergillus* or *E. coli lacZ* as 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 *Debaryomyces polymorphus* (Gellissen et al., 2005). Such a single expression system that can be used to utilize the power of a number of yeasts together with the complete genome sequence of a number of non-conventional yeasts available that allow specific pathway engineering and tweaking of several metabolic pathways to improve any given process, becomes a reality.

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