Chapter 16
Yeast Genetics and Biotechnological Applications

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Contents

Abstract Yeast can be recognized as one of the very important groups of microorganisms on account of its extensive use in the fermentation industry and as a basic eukaryotic model cellular system. The yeast *Saccharomyces cerevisiae* has been extensively used to elucidate the genetics and regulation of several key functions in the cell such as cell mating, electron transport chain, protein trafficking, cell cycle events and others. Even before the genome sequence of the yeast was out, the structural organization and function of several of its genes was known. With the availability of the origin of replication from the 2 μm plasmid and the development of transformation system, it became the host of choice for expression of a number of important proteins. A large number of episomal and integrative shuttle vectors are available for expression of mammalian proteins. The latest developments in genomics and micro-array technology have allowed investigations of individual gene function by site-specific deletion method. The application of metabolic profiling has also assisted in understanding the cellular network operating in this yeast.

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This chapter is aimed at reviewing the use of this system as an experimental tool for conducting classical genetics. Various vector systems available, foreign genes expressed and the limitations as a host will be discussed. Finally, the use of various yeast enzymes in biotechnology sector will be reviewed.

Keywords Eukaryotic model, plasmid, genomics, micro-array technology, metabolic profiling

16.1 Introduction

Yeasts are single-celled, eukaryotic, saprophytic organisms which are more complex than bacteria. For typical budding yeast such as *Saccharomyces cerevisiae*, the size of the cell may be about 3–7 μm in width and 5–15 μm in length. This may increase to 15–20 μm during cell division. However, in case of fission yeast such as *Schizosaccharomyces pombe*, the cells can elongate to 18–20 μm at the time of cell division. In terms of structural organization, the yeasts are similar to other eukaryotic cells and are characterized by the presence of nucleus, mitochondria, large vacuoles etc. The DNA in *S. cerevisiae* is organized into 16 chromosomes (haploid number). The ease with which mutants can be isolated, characterized and mapped makes it an ideal system to carry out eukaryotic genetics. The pace with which the work can be carried out is as rapid as with the bacterial systems. Some of other properties that make it useful are its non-pathogenicity, ease of cultivation, rapid growth (the doubling time on glucose is 90 min and about 3.5–4 h on a non-fermentable carbon source), application of replica plating methods, mutant isolation and a well-defined haploid and a diploid life cycle. Various stable biochemical mutants can be isolated, many of them for essential functions, and expressed in the haploid state and complementation tests carried out in diploids to study the segregation of genes. Since the products of mitotic division are held together in a 4-spored ascus, the analysis of their phenotype gives a useful tool for following segregation of markers. Baker's yeast cells are also available commercially and provide a cheap source of cells.

Two significant findings have led to revolutionary advances in application of modern molecular biological techniques. These were the 'discovery' of the 2 μm plasmid, the origin of replication of which was used for construction of numerous stable plasmid vectors. The other was the development of a transformation system in *S. cerevisiae*. During early years, a large number of structural genes from this yeast were identified from plasmid libraries by complementation analysis in bacterium *Escherichia coli.* Many of the plasmids (Integrative YIp series) could enter into yeast chromosome by homologous recombination. External DNA containing partially homologous sequences can therefore be directed to specific locations in the yeast genome. Coupled with the high levels of gene conversions noticed in yeast, these have led to direct replacement of engineered sequences into their normal chromosomal locations. In fact, a library of mutants for each of the annotated genes

was constructed at Stanford University. These single gene knock-out libraries are available through commercial sources for use by scientists.

Very recently, transformations with single synthetic oligonucleotides have been reported in yeasts allowing manipulations of genes encoding proteins. This technique has been exploited for studying gene regulation, structure- function relations, chromosome structure etc. A number of mammalian proteins are also being expressed in yeast to study their functions. With the availability of the genome sequence of *S. cerevisiae,* a new way of doing science in this yeast has emerged.

16.2 Yeasts of Biotechnological Importance

A large number of genera have been found to be of industrial importance but the most commonly used yeast has been *S. cerevisiae*. Therefore, a reference to 'yeast' is associated with this genus and species. Yeasts have been used for preparation of wine, beer and bread for thousands of years. Due to a large body of information available on its life cycle, genetics, plasmid (the 2 μm plasmid), it was the first yeast to be used for production of heterologous proteins such as interferon (Hitzeman et al., 1981), hepatitis B surface antigen (Valenzuela et al., 1982). Many other yeasts such as *Arxula adeninivorans*, *Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, S. pombe, Yarrowia lipolytica* have also been used successfully for production of heterologous proteins.

The choice of the alternate yeast systems is based on their ability to utilize cheap carbon sources. *Hansenula, Candida,* and *Pichia* have been used as hosts due to their ability to use methanol as a carbon source (Gleeson and Sudbery, 1988). C_1-compounds as sole source of carbon are utilized by *Candida boidinii* and some species of *Torulopsis* (Harder and Veenhuis, 1989; Sahm, 1977). These also use inorganic nitrogen source such as methylated amines increasing their versatility. Utilization of methanol by *Pichia* involves action by alcohol oxidase that converts methanol into formaldehyde. This is converted into carbon dioxide and water by consecutive activities of formaldehyde dehydrogenase and formate dehydrogenase. Hydrogen peroxide is also released as a by-product which is converted into water and oxygen by catalase. The promoter of methanol oxidase is inducible by methanol and repressed by glucose and ethanol which can serve to regulate induction of expression of foreign proteins. *P. pastoris* has been used for production of single cell protein (SCP) and heterologous proteins using the alcohol oxidase promoter. Some of these are described later.

Yeasts such as *Candida tropicalis*, species of *Torulopsis*, and *Y. lipolytica* have been selected based on their ability to utilize *n*-alkanes as a carbon source. Other hydrophobic substrates such as fatty acids and oils can also be utilized by *Y. lipolytica* (for review, see Fickers et al., 2005a) Some of these yeasts have been used for production of TCA cycle intermediates, vitamins and co-enzymes. The metabolism of *n*-alkanes is carried out by their conversion to fatty acids which are degraded to

CO_2 and H_2O by the β-oxidation pathway. The enzymes of this pathway are located in peroxisomes in these yeasts.

A more recently developed yeast for expression of foreign proteins is based on *Arxula adeninivorans*. In addition to being thermo- and halo-tolerant, it can grow on a number of carbon sources such as starch, tannic acid, *n*-alkanes and purines (for a recent review, see Steinborn et al., 2007a). Yeast such as *Brettanomyces naardenensis*, some species of *Pichia* and *Candida*, *Pachysolen tannophilus* are also capable of utilizing pentoses such as xylose for growth (Freer and Destory, 1983). As some such pentoses are a part of hemicellulose fraction in lignocellulosic complexes, there has been interest in use of these yeasts for biomass conversion. However, useful end products such as ethanol or acids are produced in low quantities by these yeast. Ethanol levels varying from 6.6–10.0 gl^{-1} have been obtained using *Candida shehate* and *Pichia etchellsii* (Kaur and Mishra,, unpublished data) and hence these have not been seriously considered for alcohol production. Since utilization of xylose requires additional enzyme (s), attempt has been made to express these enzymes in *S. cerevisiae* so that major fractions(hemicellulose/cellulose) of the lignocellulosics components can be utilized for alcohol production.

16.3 Genetic Material and Methods of Manipulation

The size of the yeast genome (about 15 Mbp) is about 4 times more than that of *E. coli* but unlike the latter, it is organized into 16 chromosomes. In the diploid cell, the number of chromosomes is 32. Several polyploids strains, the industrial strains, are extensively used but for general routine analysis of biochemical and genetic phenomenon, well defined yeast strains are used. Under light microscope, the nucleus is visible and is a distinguishing feature between bacterial and a yeast cell. Like most eukaryotes, the information is first processed into RNA's and then into proteins. A completely different mode of gene regulation is practiced in comparison to that seen in prokaryotes.

16.3.1 Chromosome Organization

Techniques such as pulse-field or orthogonal-field alternating gel electrophoresis have been used for the separation of the chromosomes. Each chromosome consists of a single double stranded DNA molecule (Fangman and Brewer, 1991) which is associated with histones H2, H3, and H4 to give rise to nucleosomes. Replication origins are located at an average distance of 40 kbp on the chromosome and are identical with autonomously replicating sequences (ARS). These can be identified by way of their imparting the property of autonomous replication to the plasmids harboring them. The size of the individual chromosome varies from 200–2200 kbp. Through the genome sequencing project, 6,183 open-reading frames (ORF's)

exceeding 100 amino acids were predicted out of which 5,800 were confirmed to correspond to protein-encoding genes (Sherman, 2006). The yeast genome is highly compact and 72% of DNA codes for genes. The average size of the gene is estimated at 1.45 kb, or 483 codons with a range from 40 to 4910 codons. A small proportion (3.8%) of ORF does contain introns which are predominantly in tRNA encoding genes. In spite of a large body of genetic information available for yeast, only 30% of predicted genes have been characterized experimentally. One of the most interesting features is the presence of retrotransposons, the movable DNA elements, which vary in number and position on the chromosomes in different strains of *S. cerevisiae*. The number is about 30 in common laboratory strains. The telomeres (TEL) are located at each end of the chromosome and are required for the stability of the chromosomes. These elements have been studied recently to understand their role in cell aging. Many of the chromosomal elements have been used (ARS, centromere CEN, TEL) to construct useful vectors for yeasts. Some yeasts such as *S. pombe* contain many genes with introns.

16.3.2 Extrachromosomal Materials

S. cerevisiae contains an autonomously replicating plasmid, the 2 μm plasmid, in its nucleus which is present in about 60–100 copies. The plasmid has an origin of replication but does not appear to confer any selective advantage to the host as cir^0 cells (lacking the plasmid) are indistinguishable from the cir^+ wild type cells. However, the chromosomal mutation *nib1* causes a reduction in growth of cir^+ strains, due to abnormally high copy number of the 2 μm plasmid (Sherman, 2006). While the plasmid itself has not been used for expression of foreign proteins, the vectors derived from it are extensively being used. The plasmid encodes four genes *FLP* (A), *REP1* (B), *REP2* (C) and (D), an origin of replication, STP locus (for stabilization) and two 599 bp inverted repeat sequences. FLP encodes for a site specific recombinase which promotes flipping about FLP recombination targets (FRT) within the inverted repeats and converts A form of the plasmid to B form in which gene order has been rearranged by intramolecular recombination. Therefore cells contain two forms of 2 μ, A and B (Hollenberg et al., 1976). The function of (D) is not known. Occurrence of the recombination event right after the onset of bidirectional replication leads to the replication forks pointing in the same direction. Such an event leads to formation of large number of plasmid molecules which can be resolved by another round of recombination. Other yeasts, such as *K. lactis*, some species of *Zygosaccharomyces* have been found to contain plasmids as well but these do not bear any sequence similarity to the 2 μm plasmid nor have these been used so extensively for vector construction.

Mitochondria contain the mitochondrial DNA which is 70–76 kbp in length and is present in 50 copies/cell. It has 15 genes that code for the proteins involved in the mitochondrial translational machinery, subunits of the respiratory chain and, some tRNA genes. About 15% of the mitochondrial proteins are coded for by this

DNA while the remaining are coded for by chromosomal DNA. Lack or mutations in the mitochondrial DNA lead to *rho*(ρ-) phenotype and these cells are respiratory deficient due to lack of subunits of some respiratory enzymes (cytochrome b, cytochrome c oxidase, ATPase complexes). These cells, however, are viable and still retain mitochondria, although morphologically abnormal.

All yeast strains contain double stranded (ds) RNA viruses that constitute about 0.1% of the total nucleic acids. The viruses have been categorized into three categories, L-A, L-BC, and M. Two other categories (till now considered non-viral) T and W are also found. M dsRNA encodes a toxin, and L-A encodes the major coat protein and components required for the viral replication and maintenance of M. The M and L-A dsRNA's are packaged into virus particles separately with the common capsid protein coded for by L-A. These particles are also transmitted cytoplasmically during vegetative growth and conjugation in a non-chromosomal inheritance pattern. L-B and L-C are similar to L-A, have an RNA dependent RNA polymerase and are present in intracellular particles. Lack of M dsRNA leads to *KIL*-o phenotype and these cells are readily induced by growth at elevated temperatures, and chemical and physical agents (Sherman, 2006). Yeast also contains a 20S circular single-stranded RNA that appears to encode RNA –dependent RNA polymerase that acts as an independent replicon. The segregation of this nucleic acid follows the non-Mendelian route.

16.3.3 Yeast Manipulations

The yeast *S. cerevisiae* is an ideal organism accessible to various genetic manipulations due to a well defined life cycle, development of transformation protocols, availability of suitable auxotrophic strains and availability of vector systems. All these properties have been put together for the production of foreign proteins for medical, research and industrial use. Recombinant protein expression was first described in 1981 for human α-interferon (Hitzeman et al., 1981) followed by many other examples in the subsequent years. In this section, the features required for heterologous expression of proteins in *S. cerevisiae* and some other yeast hosts are described in detail.

16.3.3.1 Molecular Transformation

Exogenously added DNA molecules can be introduced into yeast cells by three different methods which overcome the permeability barrier of thick cell wall. Molecular transformation of yeast was first achieved by a technique which involved the production of spheroplasts (wall-less yeast cells) with enzymes such as β-glucuronidase or zymolyase in an osmotically stabilized medium containing sorbitol (Hinnen et al., 1978; Beggs, 1978). The yeast spheroplasts are fused with PEG in the presence of $CaCl_2$ and DNA and finally plated out under selective conditions embedded in a top agar to facilitate regeneration of cell wall. This method is very commonly used and

is most efficient in terms of transformation frequencies per microgram of transforming DNA (10^5–10^6/μg DNA). It suffers from three disadvantages which are (i) the diploid and the triploid cells are formed by cell fusion using PEG, (ii) the screening procedure becomes difficult as the transformants are embedded in the agar overlay, and (iii) the method is time consuming and laborious. The method is significantly affected by the phase in which the cells are taken. Also, the exposure to enzyme has to be carefully monitored. Hence, this method finds application only for special purposes such as transformation with gene libraries and introduction of very large Yeast Artificial Chromosomes (YAC) based plasmids into the cell.

The more convenient methods were developed later in which the yeast cells were made competent by lithium salts (Ito et al., 1983) or use of electroporation (Becker and Guarante, 1991). Both these methods are more convenient than the spheroplast method as colonies grow on the surface of the agar plate used for selection. The former method does not give significantly any higher frequencies of transformation but is less laborious and does not require any incubation at −70°C. The electroporation method has to be optimized for each yeast species used and depends on the availability of an electroporator. An order of magnitude higher transformation frequency has been reported using this method. The freeze method has also been reported to work efficiently for *S. cerevisiae* as well as many other yeasts (Klebe et al., 1983). It has a distinct advantage in that it is independent of the yeast strain used and the cells can be used for long term. A rapid and convenient method which uses cells directly from agar plate involves agitation of cells with glass beads to introduce competence (Constanzo and Fox, 1988). All yeast strains are not equally susceptible to transformation, and the transformability of a single strain may vary from method to method.

The newly introduced DNA can meet one of the two fates inside the cell depending upon if it has an origin of replication (ORI) or not. If it contains an ORI (derived from the 2 μm plasmid) or the CEN sequence of the chromosome, it can be maintained independently in the cytoplasm. Since the incoming DNA has an auxotrophic marker, it can be maintained by growing the transformed cells on a selective medium. The only disadvantage is that in the absence of a selection pressure the plasmid is likely to be lost. In the second method, since the plasmid does not contain an origin of replication, the newly introduced DNA becomes integrated into the chromosome following homologous recombination. In such a case, the plasmid is inherited stably in spite of lack of a selection pressure. This method also finds applications in the production of heterologous proteins in yeast and will be discussed later.

16.3.3.2 Selectable Markers

16.3.3.2.1 Auxotrophic Selectable Markers

The most commonly used markers include *HIS3, LEU2, LYS2, TRP1*, and *URA3*, which complement specific auxotrophic mutation in yeast, such as *his3-Δl, leu2-Δl, lys2-201, trp1-Δl and ura3-52* (Beggs, 1978; Rose et al., 1984; Struhl et al., 1979; Tschumper and Carbon, 1980). These mutants have low reversion rates, hence give

Table 16.1 Selectable markers used in *S. cerevisiae* transformation

Markers	Marker type: dominant (D)/ Auxotrophic(A)	Comments	References
URA3	A	(a) Selection possible in casamino acid (CAA)	
		(b) Counter selection with 5- fluoro-ortic acid (5-FOA)	Boeke et al., 1989
		(c) URA3-d for high copy no. selection	Loison et al., 1989
LYS2	A	(a) Counter selection using α-amino adipate	Barnes and Thorner, 1986; Chattoo et al., 1979; Fleig et al., 1986
TRP1	A	Selection in CAA	
HIS3	A		
LEU2	A	LEU2-d for high copy no. selection	
Cm^r(Chloramphenicol-resistance) gene	D	(a) Selection using chloramphenicol in glycerol medium	Hadfield et al., 1986
		(b) Effective only using yeast promoter	
Herpes simplex virus thymidine Kinase gene [HSV TK]	D	(a) Thymidine/ Sulphanilamide/ amethopterin selection	Zealey et al., 1988
		(b) The level of resistance dependent on gene dosage	
S. pombe triose phosphate isomerase gene	D	(a) Marker used in *S. cerevisiae* tpi^- host	Kawasaki, 1986
		(b) autoselection in glucose	
Tn903 Kam^r	D	(a) Selection using G418	Hadfield et al., 1990

low background in transformation. The selection requires minimal growth media lacking the relevant nutrient. A description of selectable markers employed for *S. cerevisiae* transformation is given in Table 16.1.

LYS2 and *URA3* are versatile as both positive and negative selections are possible. The positive selection is carried out by auxotrophic complementation of the *ura3* and *lys2* mutation. Negative selection of the *lys2* and *ura3* cells is based on toxic antimetabolites a-aminoadipic acid and 5-fluoro-ortic acid respectively,

which prevent growth of the prototropic strains and allows mutants to grow (Boeke et al., 1984; Barnes and Thorner, 1986; Chattoo et al., 1979; Fleig et al., 1986). The use of these genes requires a suitably marked recipient strain. As the mutations are recessive, therefore the recipient is haploid or a specifically made homozygous diploid. Industrial strains are often polyploid and their genetics is poorly defined. A suitable recipient is difficult to obtain, therefore dominant selectable markers need to be employed when dealing with industrial yeast strains.

16.3.3.2.2 Dominant Selectable Markers

The dominant markers increase the range of host strains and can be used for selection in rich medium. Some examples are resistance to G418 conferred by Kanamycin resistance gene of Tn903 which codes for neomycin phosphotransferase II (Webster and Dickson, 1983), chloramphenicol acetyl transferase (Jimenez and Davies, 1980), hygromycin β phosphotransferase (Gritz and Davies, 1983), copper resistance conferred by copperthionein *CUP*^{R} gene (Fogel and Welch, 1982).

Autoselection: Autoselection systems have also been developed to ensure plasmid selection irrespective of culture conditions. The expression of cDNA encoding the yeast killer toxin and immunity gene could be used for self selection of transformants of industrial and research laboratory yeast since plasmid containing cells kill plasmid free cells (Bussey and Meaden, 1985).Another system used *ura3 fur1* strains as host cells for plasmids containing the *URA3* gene. Both, the *de novo* and salvage pathway for uridine 5 -monophosphate synthesis are blocked, hence these are non-viable, therefore maintenance of a *URA3* plasmid is obligatory for viability even in uracil containing media. The transformant was obtained by mating a *fur1* strain with an *ura3* strain containing the *URA3* plasmid and selecting the plasmid containing *ura3 fur1* progeny (Loison et al., 1986).

16.3.3.3 Yeast Vectors

16.3.3.3.1 Yeast Integrating Plasmids (YIp)

These are simple plasmids which do not replicate autonomously but integrate into the yeast genome by homologous recombination (Orr-Weaver et al., 1983). They lack ARS sequences and must carry at least one region homologous to a yeast chromosomal sequence. The transformation with YIp is not very efficient and < 10 transformants per μg of transforming DNA are obtained. However, the recombinant strains obtained with YIp are highly stable, even in the absence of selective pressure.

The YIp vectors integrate as a single copy. Multiple integration occurs at low frequencies. YIp plasmids with two yeast segments such as *YFGI* and *URA3* markers, can integrate at either of the two genomic loci. Repetitive DNA sequences, such as the rDNA and Ty elements in the vector can promote integration at any of the multiple sites in the genome. Multi-copy integrants are relatively stable and have

been used in gene dosage studies (Cashmore et al., 1986). These are also preferred for high level of expression of recombinant proteins. YIp is the vector of choice for the experiments in which recombinant yeast must retain the cloned gene for long periods in culture. Transplacement, an alternative type of integration, makes use of double homologous recombination to replace yeast chromosomal DNA (Rothstein, 1983). Such vectors contain the exogenous DNA and selection marker flanked by yeast DNA homologous to 3′ & 5′ regions of chromosomal DNA to be replaced. Before transformation, the vector is digested with restriction enzymes which liberate the transplacing fragment with 5′ and 3′ homologous ends.

A hybrid vector system comprising of the YIp and two terminal direct repeat (δ) sequences of the yeast retrotransposon Ty was constructed. The Ty element has two δ sequences one of which remains at the original site after transposition of the Ty retrotransposon. These sequences are distributed on each chromosome of most laboratory strains of *S. cerevisiae* and provide sites for single and multi-copy integration. Expression of several foreign proteins such as α-amylase, human nerve growth factor was reported using this system (Sakai et al., 1991). The integration was stable over a period of 50 generations under non-selective conditions.

16.3.3.3.2 Episomal Vectors (YEp Plasmids)

The vectors derived from the 2 μm plasmid are called Yeast Episomal Vectors (YEps). The copy number of these vectors varies from 10–40 per cell. Some YEp vectors contain the 2 μm Ori and the *REP3* gene whereas others contain the entire 2 μm plasmid. Plasmids of the former type must be propagated in cir^+ hosts containing the entire 2 μm plasmid as action of other genes (such as *REP1* and *REP2*) of the plasmid is required for accurate portioning of the plasmids into daughter cells. The most commonly used expression vectors are *E. coli* - yeast shuttle vectors based on the 2 μm plasmid (Armstrong et al., 1989; Broach, 1983; Parent et al., 1985). The first YEp vectors were pJDB219 and pJDB248 (Beggs, 1978). These vectors consist of the 2 μm plasmid combined with pBR322 for replication in *E. coli* and *LEU2* gene for selection. They are relatively stable and transform with high frequency of 10^4–10^5 transformants μg^{-1} DNA. PJDB207 (Beggs, 1981) and YEp13 (Broach, 1983) are smaller vectors containing only origin and STB locus. They are relatively stable and transform with high efficiencies. Many other YEP plasmids are reported to be unstable and tend to be lost at the rate of 1/100 or more cells after each generation. Under conditions of selective growth, only 60–95% of the cells retain the YEp plasmids (Sherman, 2006). Mutations in several key genes have been designed to develop high copy number plasmids of the YEp type.

16.3.3.3.3 Yeast Autonomously Replicating Plasmids (YRps)

These vectors contain the yeast autonomously replicating sequences (ARS sequence) instead of the 2 μm plasmid origin of replication and allows the transformed plasmids

to propagate several hundred folds. YRp7 is an example of such type of replicative plasmid. It is made up of pBR322 and a yeast gene *TRP1*. *TRP1* is involved in tryptophan biosynthesis and located adjacent to chromosomal origin of replication. YRps give 10^3–10^4 transformants μg^{-1} of DNA. These vectors are highly stable and present in multiple copies per cell (1–20). The plasmid free cells accumulate at a rate of up to 20%/generation without selection. This is due to inefficient transmission to daughter cells during cell division (Murray and Szoatak, 1983). The proportion of plasmid-containing cells can be very low even when grown under selection.

16.3.3.3.4 Yeast Centromere Plasmids (YCps)

Yeast ARS vectors can be made stable by addition of yeast centromeric sequences (CEN). In this case, copy number is reduced to 1–2 per cells (Clarke and Carbon, 1980). The CEN function is dependent on three conserved domains, all of which are required for stabilization of the YCp plasmids. Yeast ARS vectors are hardly ever used for foreign gene expression. However, ARS/CEN vectors are used where low level expression is required. These mimic the natural chromosomes in that they segregate to two of the four ascospores in the ascus. The low-copy number & stability of YCp vectors makes them ideal for cloning and construction of genomic DNA libraries. ARS1 is most commonly used ARS element for YCp vectors. Among the centromeres, CEN3, CEN4 and CEN11 are the ones which can be manipulated conveniently. YCp plasmids generally segregate in a Mendelian fashion during meiosis. The vector YCp50 contains ARS1 and CEN4. Some commonly used yeast transforming vectors are shown in Fig. 16.1.

Disintegration Plasmids: Disintegration vectors are also based on the 2 µm plasmid (Chinery and Hinchcliffe, 1989). The sequences necessary for propagation in bacterial cells are bound by targets for the 2 µm FLP gene product. The excision and consequent loss of these bacterial sequences occurs upon transformation of yeast cells. The expression cassette is cloned into REP1 locus of the 2 µm circle. Such constructs are completely stable and are present in high copy number. Some commonly used yeast vectors are listed in Table 16.2.

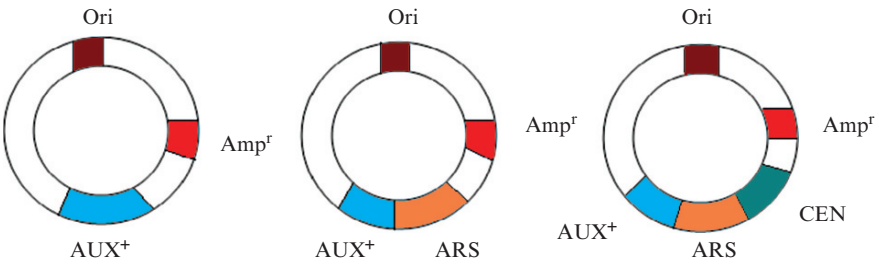

Fig. 16.1 Yeast transforming vectors. Ori: origin of replication; Amp^r: ampicillin resistant gene**;** AUX$^+$: wild type allele of yeast auxotrophic marker; ARS: autonomous replication sequence**;** CEN: centromere sequences from yeast

Table 16.2 Some commonly used *S. cerevisiae* vectors and their important features

Vector	Yeast sequences	Copy number/ cell	Transformation frequency/μg DNA	Stability	Advantages	Disadvantages	Reference
Yip	Homologous DNA	≥ 1	10^1–10^2	Less than 1%	• Provide most stable maintenance of cloned genes • Integrated YIp behave as genetic marker • Used to introduce inversions, deletions & transpositions	• Transformation frequency low	Hinnen et al., 1978
Yep (2μ based)	ORUI, STB, REP1, REP2, FLP	50–200	10^4	1%	• High copy no. plasmid • High transformation frequency • Readily recovered from yeast cells • Useful for complementation studies	• Novel recombinants can generate in vivo by recombination with endogeneous 2μm plasmids	Futcher and Cox, 1984
YCp	ARS/CEN	1–2	10^2–10^4	Less than 1%	• High transformation frequency • Useful for complementation studies • Show Mendelian segregation at meiosis • Low copy no is useful if product of gene is deleterious to cells	• Low copy no. • Recovery of vector is more difficult than YEp & YRp plasmids	Clarke and Carbon, 1980
YRp	ARS	1–20	10^3–10^4	20%	• High copy no. • Readily recovered from yeast	• Transformants are unstable	Murray, 1987
Ty/YIp	Ty & DNA	Depends on the vectors used to introduce Ty into the cell	≤ 20	Stable	• Amplification following chromosomal integration	• Needs to be introduced into cell in another vector	Sakai et al., 1991; Shuster et al., 1990

YAC	TEL, ARS, CEN	Stability depends upon length longer the YAC is more stable it is	• Very long DNA molecules > 40 Kb can be cloned	• Difficult to map by standard techniques	Sambrook and Russell, 2001

16.3.3.4 Yeast Artificial Chromosomes (YACs)

YACs are specialized vectors capable of accommodating extremely large fragments of DNA (100–1000 kb). Libraries of mammalian genomic DNA have been constructed that contain over 1 Mb of DNA (Sambrook and Russell, 2001). The architecture of these vectors mimics that of the eukaryotic chromosome in that it contains an ARS, a centromere, two telomeres and two yeast selectable markers separated by unique restriction sites. Each arm also contains a nutritional marker and an appropriately oriented DNA sequence that functions as a telomere. One of the two arms contains the ARS sequence. These also contain sequences for replication and selection in *E. coli*. YACs are linear molecules when propagated in sequences between the tips of the telomere for propagation in bacteria. Large YAC constructs are as stable as natural chromosomes. The transformants containing the YAC can be identified by plating on minimal medium. The insertion of foreign DNA into the cloning site interrupts a tRNA suppressor gene resulting in the formation of red colonies in strains carrying an ochre mutation in the *ADE2* gene. The sequences which are not possible to clone in *E. coli* cosmid and Lambda vectors can be cloned successfully in YAC vectors. They are powerful vectors for construction of genomic DNA libraries even from complex genomes such as human genome (Burke et al., 1983). The important components of the YAC are shown in Fig. 16.2.

16.3.3.5 Yeast Expression Vectors

Yeast expression vectors employ a number of promoters and terminators in addition to gene of interest in the vector backbone.

Yeast promoter: The first requirement for an expression vector is an efficient promoter. Table 16.3 lists some of the promoters which are in common use for expression of heterologous proteins in *S. cerevisiae.* Constitutive promoters are derived from the genes of glycolytic pathway such as alcohol dehydrogenase (*ADH1*), pyruvate kinase (*PYK1*), phosphoglycerate kinase (*PGK1*) and enolase (*ENO*). They lead to high level of transcriptional expression. On the other hand, regulated promoters (some derived from galactose utilizing pathway, acid phosphatase) can be controlled by controlling the availability of certain nutrients. Some heterologous promoters have also been found to be effective in *S. cerevisiae*. Downstream of the promoter there must be convenient sites for restriction enzymes for insertion of genes to be expressed. There is no particular difficulty in finding a suitable poly-linker, though an A-rich environment should be sought (Kingsman et al., 1985).

Yeast terminator: Transcriptional termination of yeast mRNA is less well understood than in bacteria and higher eukaryotes. It appears that yeast mRNA follows the same pattern of termination, processing and polyadenylation of pre-mRNA as higher eukaryotes. In yeast, these processes are tightly coupled and occur within a short distance near the 3′ end of the gene (Butler et al., 1990). A number of consensus sequences have been implicated as part of mRNA terminator, especially the tripartite sequence TAG.

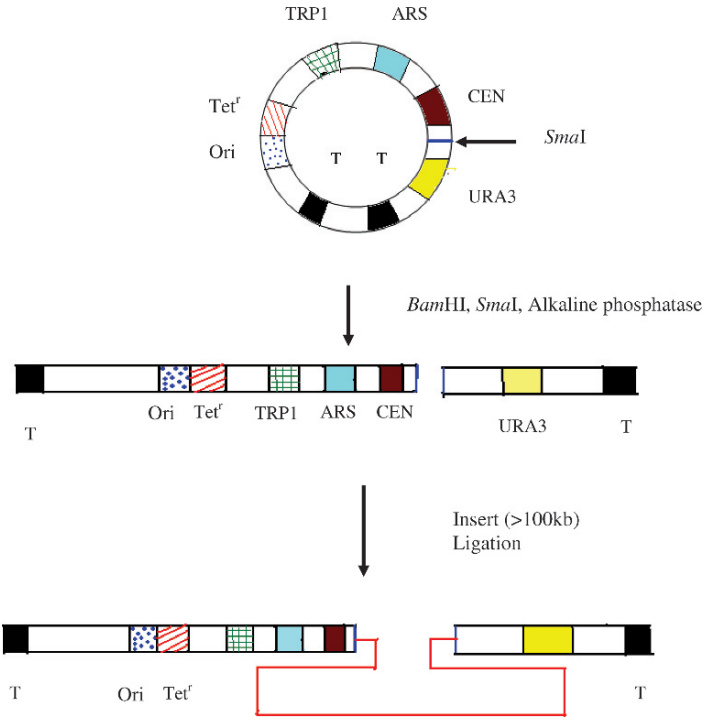

Fig. 16.2 YAC cloning system- pYAC has *E.coli* ori and selectable marker gene (Tet^r); and yeast DNA sequences TRP1, URA3, ARS and CEN. T represents telomeric sequences. URA3 is a gene in uracil biosynthesis pathway and TRP1is a gene of Tryptophan biosynthesis pathway

(T-rich)..TA (T) GT..(AT-rich)..TTT (Zaret and Sherman, 1984) and TTTTTATA (Henikoff and Cohen, 1984). Terminators from a number of genes have been used in expression vectors, e.g. *ADH1, CYC1, GAP, MF1*, *TRP1,* etc. (Urdea et al., 1984; Mumberg et al., 1995; Hitzeman et al., 1981; Rosenberg et al., 1984; Brake et al., 1984). Table 16.4 lists the eukaryotic therapeutic proteins expressed in *S. cerevisiae.*

16.4 Expression of Proteins in Non-*S. cerevisiae* Yeasts

In many cases, *S. cerevisiae* expression system is disadvantageous for large-scale production of many foreign proteins. The major drawbacks were the lack of very strong, tightly regulated promoters, need for fed-batch fermentation for attaining high cell densities and hyper-glycosylation of proteins. Therefore, other yeast expression systems have been developed. Some of the most commonly used ones are described below.

Table 16.3 Promoter systems used for expression of heterologous proteins in *S. cerevisiae*

Promoter	Gene	Protein encoded	Regulation	Strength	References
Constitutive	*ADH1*	Alcohol dehydrogenase1		+++	Hitzeman et al., 1981; Bennetzen and Hall, 1982
	PYK1	Pyruvate kinase	20-fold induced by glucose	+++	Burke et al., 1983
	PGK1	Phosphoglycerate kinase		++++	Tuite et al., 1982; Dobson et al., 1982
	ENO	Enolase	10-fold induced by glucose		Holland et al., 1981
Regulated	*ADH2*	Alcohol dehydrogenase 2	1000 fold-induced by galactose	++	Johnston and Davis, 1984
	GAL1.10.7	Galactose metabolic enzymes	100-fold repressed by glucose	+++	
	GALS	Galactokinase variant	-	+++	Mumberg et al., 1995
	MET25	O-acetyl homoserine sulphydrylase	200-fold repressed by phosphate	+	
	CUP1	Copper metallothionein	20- fold induced by Cu^{2+}	+	Karin et al., 1984
	PHO5	Acid phosphatase	100 to 200-fold induction with inorganic phosphate	++	Meyhack et al., 1982; Kramer et al., 1984
	tetO-CYC1	Tetracycline promoter	1000-fold induction with tetracycline	+++	Gari et al., 1997
Heterologous	*CaMV*	Cauliflower mosaic virus 35S promoter	RAS/cAMP pathway		Ruth et al., 1992
	ARE	Androgen response element	Dihydrotestosterone/testosterone		Eldridge et al., 2007

Table 16.4 Eukaryotic therapeutic proteins expressed in *S. cerevisiae*

Recombinant protein	Commercial name	Company	Therapeutic indication
Recombinant blood factor			
Hirudin/lepirudin	Refludan	Hoechst Marion Roussel (US)	Anticoagulant for heparin-associated thrombocytopenia
Hirudin/desirudin	Revasac	Canyon pharmaceuticals	Prevention of venous thrombosis
Recombinant hormone			
Insulin	Novolog	Novo Nordisk	Diabetes mellitus
Insulin	Exubera	Pfizer(New York) Aventis(Kent, UK)	Diabetes mellitus
Insulin	Apidra	Aventis (Germany)	Diabetes mellitus
Insulin	Liprolog	Eli Lilly	Diabetes mellitus
Somatotropin	Valtropin	Biopartners	Growth disturbances in children and adults
Glucagon	Glucagen	Novo Nordisk	Hyperglycemia
Recombinant enzyme			
r urate oxidase	Fasturtec	Sanofi-Synthetalco	Hyperuricemia
Recombinant Vaccine			
Hepatitis B	Ambirix	Glaxo Smith Kline	Immunization against hepatitis A and B
Hepatitis B	Pediarix	Glaxo Smith Kline	Immunization against hepatitis B
Hepatitis B	HBVAXPRO	Aventis Pharma	Immunization against hepatitis A and B
Hepatitis B	Infanrix-Penta	Glaxo Smith Kline	Immunization against diphtheria, tetanus, pertussis, polio and hepatitis B
Hepatitis B	Procomvax	Aventis Pasteur	Immunization against *H. influenzae* type b and hepatitis B
Hepatitis B	Primavax	Aventis Pasteur	Immunization against diphtheria, tetanus and hepatitis B
Hepatitis B	Twinrix	Glaxo SmithKline	Immunization against hepatitis A & B

16.4.1 Pichia pastoris

The most extensively developed system is based on *P. pastoris*. Molecular genetics method for *P. pastoris* like transformation, gene replacement, gene targeting and cloning by complementation are similar to the methods described for *S. cerevisiae.* This methylotrophic yeast has two key advantages over *S. cerevisiae* as a host for foreign

protein production (Cregg et al., 1993). Firstly, the promoter used to transcribe foreign gene is derived from methanol regulated *P. pastoris* alcohol oxidase 1 gene (*AOX1* promoter). In cells exposed to methanol as a sole source of carbon, transcription initiation at *AOX1* promoter is highly efficient and comparable to that of promoters which are derived from highly expressed glycolytic pathway genes (Waterham et al., 1997). Unlike glycolytic promoters, *AOX1* promoter is tightly regulated and highly repressed under non-methanol growth conditions. The second advantage of *P. pastoris* is that it is not a strong fermenter like *S. cerevisiae*. During fermentation, *S. cerevisiae* produces ethanol extracellularly, which in high density cultures, can rapidly build to toxic levels. This is called as Crabtree effect. *P. pastoris* strains are relatively easy to culture at cell densities of 100 g l^{-1} dry cell weight or greater (Siegel and Brierley, 1989). Strains have been developed for large-scale high yield production of single cell proteins using defined medium containing methanol (Wegner, 1983). Various process parameters such as pH, aeration, carbon source, feed rate likely to affect protein productivity, can be controlled in this yeast. It can also carry out post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing, similar to higher eukaryotic systems. Proteins which can not be expressed in bacteria or *S. cerevisiae*, such as G protein-coupled receptors, have been expressed in this system (Cereghino et al., 2002). Various aspects of the *P. pastoris* expression system have been described. The detailed protocols describing the construction of *P. pastoris* expression strains and foreign gene expression in this system have been given in the Invitrogen *Pichia* expression manual or readers are referred to Higgins and Cregg (1998). All *P. pastoris* expression vectors are shuttle vectors and some of the commonly used expression vectors are listed in Table 16.5.

Various factors affect the level of expression when *P. pastoris* integration vectors are used. In the first report of foreign gene expression in *P. pastoris*, higher proportion of immunogenic Human Hepatitis B surface antigens were produced in Mut^s (methanol-utilization slow) compared to Mut^+, even though expression levels were similar in both cases (Cregg and Madden, 1987). The various parameters affecting expression of tetanus toxin fragment C were investigated and equal levels were produced in Mut^+ and Mut^s hosts even when levels reached to 28% of total cell protein (Clare et al., 1991). The levels of expression were also not dependent on the type (single vs double cross-over) and the site of integration (*AOX1 vs HIS4*). A Mut^s integrant expressed *Bordetella pertussis* pertactin at 2% of total cell protein in shake flasks and 10% in fermenters, while a Mut^+ strain gave equal levels (5–6%) in shake flasks and fermenters (Romanos et al., 1991).

A combination of the *AOX1* promoter with *GAP* (glyceraldehyde 3-phosphate dehydrogenase) promoter in a strain expressing human granulocyte-macrophage colony-stimulating factor (hGM-CSF) resulted in a 2-fold increase in the production (180 mg/l) of recombinant protein (Wu et al., 2003b).The second alcohol oxidase promoter *AOX2* has also been used for production of recombinant human serum albumin (80 mg/l) by induction with 0.01% oleic acid (Kobayashi et al., 2000). Some other promoters that have been used are of the genes *FLD1* (formaldehyde dehydrogenase) and *ICL1* (isocitrate lyase). FLD1 is a key enzyme in metabolic pathway of methanol assimilation, is a tightly regulated promoter (like the *AOX1*)

Table 16.5 Commonly used *P. pastoris* expression vectors and their important features

Vector	Comments	Marker	Reference
pA0815	Expression cassette is between *Bam*HI & *Bgl*II for generation of multi copy expression vector	HIS4	Thill et al., 1990
pPIC3K	MCS for foreign gene expression; G418 selection for multicopy strains	Kan^r	Scorer et al., 1993b
pHIL-D2	*Not*I sites are present for *AOX1* gene replacement	*HIS4*	Sreekrishna, 1993
PHW 010	Constitutive promoter *GAP* controls the expression	*HIS4*	Waterham et al., 1997
pP1C9K	*AOX1* is fused to α-MF prepro signal sequence; *Not*I, *Sna*I1, *Eco*RI, *Xho*I, *Avr* II restriction sites for foreign gene insertion	*HIS4* & Kan^r	Scorer et al., 1993b
pGAP	*GAP* promoter fused to α-MF pre-pro signal sequence	ble^r	Invitrogen (Carlsbad CA)
pPICZα	MCS for cloning, *AOX1* promoter fused to α-MF pre-pro signal sequence	ble^r	Higgins and Cregg, 1998

and has good transcription efficiency (Shen et al., 1998). Nitrogen source also influences expression of proteins that are linked to the methanol dependent *FLD1* promoter. Higher expression levels of 19 U/ml were reported for *Rhizopus oryzae* lipase in *P. pastoris* which were higher than when only methanol and ammonium were used (Resina et al., 2004). While expression of dextranase using the *ICL1* promoter has been reported (Menendez et al., 2003), more work needs to be done to establish the usefulness of this promoter in *P. pastoris* expression system.

P. pastoris is capable of secreting high levels of foreign proteins. *S. cerevisiae SUC2* gene product invertase accumulated to 2.5 g l^{-1} in the medium (Tschopp et al., 1987b). In terms of post-translational modification, it is a better host for heterologous protein production than S. *cerevisiae* since the secreted products have been found to contain shorter (8–15) N-linked carbohydrate side chains than those secreted in *S. cerevisiae* (> 45). In addition to this, secreted proteins didn't contain terminal α1,3-mannose linkage (Trimble et al., 1991) which are present in *S. cerevisiae* and have been known to be immunogenic (Ballou, 1970). About 35% of N-linked oligosaccharides of endogenous glycoproteins from *P. pastoris* have less than 14 mannose residues (Grinna and Tschopp, 1989). Some proteins including bovine lysozyme (0.55 g l^{-1}) aprotonin (0.93 g l^{-1}) human serum albumin (3 g l^{-1}) and mEGF (0.45 g l^{-1}) have been secreted at high levels from *P. pastoris* (Digan et al., 1989; Thill et al., 1990; Clare et al., 1991).

Another advantage of *Pichia* system is ease of high-density growth and scale up with reduction in specific productivity (Cregg and Madden, 1987). This is important as concentration of secreted products in the medium increases with an increase in cell density. Since recombinant proteins produced in heterologous hosts can be proteolytically unstable, several process strategies can be applied to overcome this.

This includes altering the pH of the medium upon induction e.g. raising the pH to 6.0 (Clare et al., 1991b) or lowering the pH to 3.0 (Brierley et al., 1994; Koganesawa et al., 2002), providing amino acid rich supplements, lower cultivation temperature (which seems to favor protein stability), lowering specific growth rate, addition of protease inhibitors or a combination of these parameters. Several of these have been reviewed recently (Macauley-Patrick et al., 2005).

One of the major challenges using this expression system is the production and recovery of the membrane proteins which are largely hydrophobic. We have also successfully expressed extracellularly large (~90 kDa) cell wall bound proteins of related yeast in this system (Baranwal and Mishra, unpublished results).

16.4.2 Hansenula polymorpha

Apart from *P. pastoris*, methylotrophic yeast, *H. polymorpha* has also been used for heterologous protein expression. Several groups have developed a successful transformation system for *H. polymorpha* using *LEU2* and *URA3* genes from *S. cerevisiae* (Gellisson et al., 1991; Roggenkamp et al., 1986). The plasmid containing these markers complements a *leu H. polymorpha* mutant defective in enzyme β-iso-propylmalate dehydrogenase coded by *LEU2* in *S. cerevisiae.* The gene encoding methanol oxidase (*MOX*) was isolated and the promoter used to express foreign gene (Ledeboer et al., 1985). During growth on methanol, the enzyme accounts for 30–40% of total cell protein which is sequestered in peroxisomes (Giuseppin et al., 1988). In *P. pastoris* there is an absolute requirement for methanol in order to obtain significant expression of gene, however expression in *H. polymorpha* gene is de-repressed significantly during glucose limitation or in the absence of glucose (Eggeling and Samm, 1978; Egli et al., 1980). Therefore tight regulation of promoter is lost in the conditions used for high biomass fermentations (Gellisson et al., 1991). This expression system has been extensively reviewed (Gellissen, 2000; Kang and Gellissen, 2005).

A CoMed vector system has been recently reported (Steinborn et al., 2006) containing the pCoMed basic vector for integration of ARS, selection markers, rDNA sequences and expression cassettes. Various modules can be integrated in this system. The rDNA elements derived from *A. adeninivorans* and *H. polymorpha* clusters were assessed for their suitability as targeting sequences. The different combinations of the ARS, rDNA regions, selection markers and the expression cassettes have been reviewed (Steinborn et al., 2006). This gives the user flexibility to try a number of hosts simultaneously.

16.4.3 Kluyveromyces lactis

K. lactis is another well accepted system for production of foreign proteins of human use as it has been used in food industry for several years for production of β-galactosidase. It can grow on cheap substrates like lactose. This further increases

its potential as a host for production of foreign proteins, especially for low-value products. Other advantages are its faster growth and lack of hyperglycosylation of proteins (Hsieh and Da Silva, 1998).

S. cerevisiae ARS and 2 μm do not replicate in *K. lactis*, therefore transformation systems were developed by isolating *K. lactis* ARS (Das and Hollenberg, 1982; Sreekrishna et al., 1984). In *K. lactis* killer strains, two cytoplasmic linear plasmids k1 (8.9 kbp) and k2 (13.4 kbp) are present. They are stably maintained at 100–200 copies per cell. They have been considered as potential vector system. The region which encodes killer toxin in k1 can be deleted without affecting maintenance (Stark et al., 1990). By fusing markers like *LEU2* to k1 promoter, recombinant stable linear plasmids have been generated (Kamper et al., 1989; Tanguy-Rougeau et al., 1990). While this system can be used for foreign gene expression, the k1/k2 promoters are weak.

High copy number stable expression vectors of *K. lactis* have been constructed. They are based on *Kluyveromyces drosophilarum* plasmid, pKD1 which is very much similar to the 2 μm plasmid of *S. cerevisiae*. Vectors based on pKD1 have been constructed which show similarity to 2 μm vector (Bianchi et al., 1987). Various promoters have been used in *K. lactis* expression vectors e.g. *LAC4, GAL1, GAL7, GAL10, GAP*, alcohol dehydrogenase and *S. cerevisiae PGK* and *PHO5*.

A number of foreign proteins have been efficiently secreted in *K. lactis*. Prochymosin, which was poorly secreted in *S. cerevisiae* was efficiently secreted by *K. lactis* in soluble form using single copy integration vector (Van den Berg et al., 1990). Approximately 80% of the protein was secreted without a signal peptide, however, highest levels were obtained when native leader peptide or the α-factor pre-pro sequences from *K. lactis* or *S. cerevisiae* were used. The product is used on commercial scale for manufacturing various milk products. Using pKD1-derived vector, secretion of Human serum albumin (HSA) was also described (Fleer et al., 1991). Highest expressing strains produced 300 mg l^{-1} of protein in shake flasks using *S. cerevisiae PGK* promoter. High density fed batch fermentations were used to produce several g l^{-1} of HSA. Several other promoter systems, such as *K1ADH4*, have been used for ethanol-dependent production of HSA (Saliola et al., 2004). HSA-CD4 fusion protein, a potential therapeutic agent against HIV infection has also been produced using this system. The secretion of interleukin IL-1β has been reported by *K. lactis* (Fleer et al., 1991). Tissue specific inhibitor of metallo-proteinases (TIMP) and variants of tissue plasminogen activator (tPA) were secreted using secretion signal (Yeh et al., 1990).

A number of hydrolytic enzymes have been expressed in this system (Bergquist et al., 2002). Other enzymes include laccases from various white rot fungi (Piscitella et al., 2005). When compared to the production in *S. cerevisiae*, the recombinant product from *K. lactis* was secreted more efficiently. Another species, *Kluyveromyces marxianus,* has also been explored as a host for expression of heterologous proteins (Pecota and Da Silva, 2005) based on its short generation time and high growth rate at elevated temperature. A number of foreign genes have been expressed successfully in this yeast (Almeida et al., 2003; Ball et al., 1999; Bartkeviciute et al., 2000; Bergkamp et al., 1993; Pecota and Da Silva, 2005). Recently, a number of thermo-stable

cellulase genes were simultaneously successfully expressed in this organism under the control of high-expression promoters (Hong et al., 2007).

16.4.4 Schizosaccharomyces pombe

The fission yeast, *S. pombe* is well characterized and an intensely studied yeast. Its life cycle and growth characteristics are very well suited for genetic and biochemical analysis. It is genetically tractable and a number of fission-yeast specific plasmids have been developed to aid molecular manipulation of this yeast. Transformation of *S. pombe* was described using lithium salts (Heyer et al., 1986), spheroplasts (Beach and Nurse, 1981) and electroporation (Hood and Stachow, 1990). A very efficient method made use of lipofectin to enhance uptake of DNA by spheroplasts (Allshire, 1990). *S. pombe* expression vectors contain sequences from *S. pombe* ARS1 or from the 2 μm plasmid of *S. cerevisiae*. The ARS vectors have copy number of about 30/cells. Stability and copy number of ARS vector is enhanced by *S. pombe* derived STB sequences (Heyer et al., 1986).

A number of heterologous proteins have been expressed in *S. pombe*. Active factor XIIIa was produced at 2 mg l^{-1} using high copy number, alcohol dehydrogenase expression vector (Broker and Bauml, 1989). Epoxide hydrolase, human liver microsomal enzyme were expressed and isolated from microsomal fraction of the yeast (Jackson and Burchell, 1988). The expression of functional single-chain antibody molecules (Davis et al., 1991), large polyprotein of infectious bursal disease virus (Strasser et al., 1989), bacterio-opsin (Hildebrandt et al., 1989), xylose isomerase (Chan et al., 1986) and β-glucuronidase (Pobjecky et al., 1990) have been described. *S. pombe* galactosylates glycoproteins like acid phosphatase (Dibenedetto and Cozzani, 1975) and invertase (Moreno et al., 1985) have also been described. Ubiquinone, a component of the electron transfer system in many organisms, has been successfully expressed in this yeast (Zhang et al., 2007). The recombinant yeast could be cultivated under high-cell-density fermentations leading to production of the protein to the level of 23 mg l^{-1}. Plasmid stability was also maintained at high level throughout the fermentations.

16.4.5 Yarrowia lipolytica

Y. lipolytica is a dimorphic yeast which is unicellular in minimal medium containing glucose or n-hexadecane and forms mycelia in minimal medium containing olive oil and casein and gives a mixture of both forms in complex medium. It is very useful in various industrial processes like bioconversions of alkane and fatty acids to alcohols, production of secondary metabolites (citric acid, mannitol etc.) and production of SCP from n-paraffins. The yeast also secretes high molecular weight

proteins like lipases, proteases, ribonuclease and an alkaline extracellular protease. The inherent property of secretion has made it a subject of intense research for foreign gene expression. This yeast combines the facility of single cell use, high secretion abilities and availability of efficient tools for post-translational modifications. Multi-copy strains have been constructed facilitating large–scale production of foreign proteins. Mutant stains lacking extracellular proteases and lipases have also been constructed enabling high protein purity in the extracellular supernatant (Fickers et al., 2005b; Nicaud et al., 2002; Pignede et al., 2000a).

Transformation of *Y. lipolytica* was first achieved with lithium acetate which permeabilized the yeast cells (Davidow et al., 1985). The homologous *LEU2* gene was used to transform the cells. Vectors containing random *Y. lipolytica* genomic fragments inserted into the upstream region of *S. cerevisiae LYS2* gene for selection of transformed spheroplasts were also used (Gaillardin et al., 1985). In addition to *LEU2*, many other genes of this yeast have been used as markers, e.g. *LYS1, LYS5, ADE1, HIS1* and *URA3*. Several plasmids for gene expression and secretion have been developed for this yeast (Nicaud et al., 2002; Madzak et al., 2004). Alkaline extracellular protease pre-pro sequences have been used for secretion of bovine prochymosin (Franke et al., 1988) and porcine α-interferon (Heslot et al., 1990; Nicaud et al., 1991).

Proteins from viruses and different organisms, ranging in size from 6 to 116 kDa, have been expressed successfully in this yeast (Madzak et al., 2005).

16.4.6 Arxula adeninivorans

A. adeninivorans, a non-conventional dimorphic thermo- and salt-resistant yeast has been developed as a host for heterologous gene expression. The yeast is able to assimilate and ferment many compounds as sole source of carbon and/or nitrogen. It utilizes n-alkanes and degrades starch efficiently. *A. adeninivorans* is able to grow at cultivation temperatures of up to 48°C in media containing up to 20% NaCl. Additionally, the dimorphism of the yeast is unusual. *Arxula* grows at temperatures of up to 42°C as budding cells and turns into mycelia at higher temperatures. When the cultivation temperature is decreased below 42°C, the dimorphism is reversed and budding is re-established (Wartmann and Kunze, 2000). Alteration of morphology correlates with changes in secretion behavior. Mycelial cultures accumulate two-fold higher protein concentrations and contain two- to five-fold higher glucoamylase and invertase activities in the medium than budding cells. Based on these unusual properties, *A. adeninivorans* has been used for heterologous gene expression and as a gene donor to construct more suitable yeasts for biotechnology. Glucoamylase gene of *A. adeninivorans* was successfully expressed in *K. lactis* and *S. cerevisiae*.

Transformation system is used for heterologous gene expression. The transformants obtained are mitotically stable. Some of the unusual biochemical properties support its usage for production of many recombinant proteins. This

system primarily leans on integration vectors containing an expression cassette which contains the gene for the heterologous protein, a suitable selection marker and the rRNA targeting sequences. The basic *A. adeninivorans* transformation/expression vector pAL-HPH1 has been equipped with the *E. coli*-derived *hph* gene, conferring hygromycin B resistance, and the 25S rDNA from *A. adeninivorans* for rDNA targeting. Recombinants are based on the integration of linearized DNA fragments in 2–10 copies, e.g. into the 25S rDNA of *A. adeninivorans* by homologous recombination. Transformants were obtained for both budding cells and mycelia. In both cell types, similar expression levels were achieved and the green fluorescent protein was localized in the cytoplasm while more than 95% of the HSA accumulated in the culture medium (Wartmann et al., 2001). The undesired use of toxic hygromycin B for selection of the transformants forced the development an *ALEU2/AILV1* selection system. However, these were reported to be unstable.

Recently, a host/vector expression system based on *atrp1* gene disruption mutant has been developed. This host was transformed with the plasmid pAL-ATRP1-amyA (containing the *ATRP1* gene as the selection marker, 25S rRNA sequences for targeted insertion). Amylase (amyA of *Bacillus amyloliquifaciens*) production served as a marker for assessment of protein production. Good product levels were detected in the culture medium (Steinborn et al., 2007a) by integration of a single copy of the amylase gene. A fused vector element (consisting of the ATRP1 selection marker fused to a newly generated truncated ALEU2 promoter of 53 bp) allowed for multiple insertion of the vector containing the amyA protein. This resulted in superior productivity of the secreted recombinant α-amylase (Steinborn et al., 2007b). Clearly, a number of different strategies or a combination of these can be used to achieve higher expression of foreign proteins.

A partial list of expressed foreign proteins is given in Table 16.6 to provide the reader an idea of types of proteins that can be expressed using non- *S. cerevisiae* yeasts.

16.5 Industrial Enzymes

Over 500 different enzymes covering about 50 applications as in detergents, wine and beer making etc. are currently used in various industrial sectors. Due to different methods available in protein engineering, the scope of using enzymes is increasing. The enzymes can be grouped into three categories (Cherry and Fidantsef, 2003). The largest, comprising 65% of the sales are technical enzymes and include enzymes used in detergents, starch, textile, leather, pulp and paper and personal care products. The next segment (25%) is of food enzymes and includes enzymes used in dairy, brewery, wine juices, fats and oils, baking industries. The next (10%) are the feed enzymes. Today, over 90% of the enzymes are produced by recombinant organisms to maximize product purity and economy of production. As much as 40 g l^{-1} concentrations can be achieved.

Table 16.6 Partial List of foreign proteins expressed in non-*Saccharomyces* yeasts

Protein	Location	Promoter	References
P. pastoris			
HSA	Secreted	*AOX1*	Sreekrishna et al., 1989
Human EGF	Secreted	*AOX1*	Brierley et al., 1994
Murine EGF	Secreted	*AOX1*	Clare et al., 1991
S. cerevisiae invertase	Secreted	*AOX1*	Tschopp et al., 1987
Bovine lysozyme	Secreted	*AOX1*	Digan et al., 1989
Aprotinin	Secreted	*AOX1*	Thill et al., 1990
β-galactosidase	Intracellular	*AOX1,* DHAS	Tschopp et al., 1987
HBsAg	Intracellular	*AOX1*	Cregg and Madden, 1987
Tetanus toxin fragment C	Intracellular	*AOX1*	Clare et al., 1991
Pertactin	Intracellular	*AOX1*	Romanos et al., 1991
Streptokinase	Intracellular	*AOX1*	Hagenson et al., 1989
TNF	Intracellular		Sreekrishna et al., 1989
Hansenula polymorpha			
HBsAg	Periplasmic	*MOX, FMD*	Shen et al., 1989; Janowicz et al., 1991
PreSl -S2-HBsAg	Periplasmic	*MOX*	Janowicz et al., 1991
Human serum albumin	Secreted	*FMD*	Hodgkins et al., 1990
Glucoamylase	Secreted	*FMD*	Gellisson et al., 1991
Kluyveromyces lactis			
HSA, HSA-CD4	Secreted	*LAC4, S. cerevisiae PHO5, S. cerevisiae PGK*	Fleer et al., 1991
Prochymosin	Secreted	*LAC4*	Van den Berg et al., 1990
Schizosaccharomyces pombe			
Factor XIIIa	Intracellular	*adh*	Broker and Bauml et al., 1989
IBD virus VP3	Intracellular	*adh*, *S. cerevisiae ADH1*	Jagadish et al., 1990
Single chain antibody	Intracellular	*adh*	Davis et al., 1991
Antithrombin III	Secreted	*S. cerevisiae ADH1, S. cerevisiae CYC1*	Broker et al., 1987
Yarrowia lipolytica			
Bovine prochymosin	Secreted	*XPR2, LEU2*	Franke et al., 1988
Porcine IFN	Secreted	*XPR2*	Heslot et al., 1990; Nicaud et al., 1991
S. cerevisiae invertase	Secreted	*XPR2*	Nicaud et al., 1991

16.5.1 Yeast as a Source of Industrial Enzymes

Yeasts have great potential for production of microbial enzymes in the food and related sectors as this is considered as a GRAS organism and has been used for human consumption for a long time. These are unicellular, fast growing and can be

cultivated in reactors much like the bacterial cells. These can grow in simple media where growth can be monitored by simple optical density measurement method allowing for an in-depth study of growth, mass-energy balance studies and product formation kinetics.

Yeasts produce a large number of hydrolytic enzymes such as glucouronidase, glucosidases, lipases, xylanases, pectinases. Several genera such as *Candida, Saccharomyces, Pichia, Schizosaccharomyces, Kluyveromyces* are known to produce β–glucosidases. The enzymes are used as a supplement for cellulose hydrolyzing enzymes. These have also been reported (Pandey and Mishra, 1997; Bhatia et al., 2002b; Wallecha and Mishra, 2003) to carry out synthetic reaction leading to formation of glycoconjugates and will be discussed in the next section. Lipases are serine hydrolases and can be divided into following four groups according to their specificity in the hydrolytic reactions: substrate specific lipases, fatty acid specific lipases, regio- and stereo-specific lipases. They are useful for their hydrolytic as well as synthetic activities. Large amounts (nearly 1000 tonnes) of lipases are used in the detergent industry for removal of oil-based stains. Several synthesized structural lipids find application in infant formula and nutraceuticals. In the last decade, lipases are also finding a number of applications in the manufacture of pharmaceuticals, pesticides, single cell protein, biosensor preparation and in waste management. Lipases belonging to the genus of *Candida* (*Candida antarctica*, *Candida cylindracea* ATCC 14830, *Candida lipolytica*, *Candida rugosa*) and *Geotrichium candidum* are used as source of industrial lipases. *C. rugosa* lipase is one of the most extensively used lipase in the industry (Redondo et al., 1995). The steryl lipase activity of yeast *C. rugosa* finds applications for hydrolysis of steryl esters, resin acids found in wood, which would otherwise have negative impact on paper machine run ability and quality of paper. For a recent review of yeast lipases, the reader is referred to Vakhlu and Kour (2006).

Many yeasts are also reported to produce xylanases. These are *Candida guilliermondii*, *Cryptococus adeliae, Cryptococcus albidus, Filobasidium floriforme, Trichosporon cutaneum* SL 409. The pH optimum of these ranges from 4.5–5.5 and none of these is thermophilic. This low pH optimum makes it very unsuitable for application in pulp and paper sector. An extracellular acetyl esterase was isolated from *C. guilliermondii* which exhibited maximum activity at pH 7.5 and 50–60°C (Basaran and Hang, 2000) but this is more of an exception for the yeast enzymes. Some species of *Pichia* and *Kloeckera* have also been reported to produce low quantities of xylanases. Many yeasts belonging to the genera of *Debaryomyces* (*Debaryomyces hansenii*), *Hancornia* (*Hancornia speciosa*), *Stephanoaseus* (*Stephanoaseus smithiae*), *Kluyveromyce*s (*Kluyveromyces wickerhamii*) produce pectinolytic enzymes (da Silva et al., 2005). Several classes are reported under this category such as lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2), polygalactouronase (EC 3.2.1.15 and EC 3.2.1.67). Pectinases are of major importance in beverage industry as these improve pressing and clarifications of fruit juices. Some enzymes are also used in production of wine, extraction of olive oil and fermentation of tea, coffee and cocoa.

16.5.2 β-Glucosidases from Pichia etchellsii

P. etchellsii is a yeast of oenological origin and produces multiple inducible β-glucosidases. While good ethanol production has been observed by this yeast on glucose, cellobiose and xylose, its use has been limited due to restricted knowledge of many of its enzymes. When grown on cellulase hydrolyzed Avicel cellulose, the yeast was found to produce ethanol equal to the expected theoretical yield (unpublished data). One of the advantages is that the temperature for saccharification and growth of the fungus can be adjusted as the yeast grows well between 40–50°C. The efficient wall bound β-glucosidases produced by this yeast aid in conversion of cellobiose and probably other short-chain cellodextrins to glucose. The sugars are then directly converted into ethanol.

Two wall bound inducible β-glucosidases, BGL I and BGL II, were purified from this yeast which exhibited differential substrate specificity on *p*-nitrophenyl–D-glucoside (*p*NPG) and cellobiose (Wallecha and Mishra, 2003). While the internal peptide sequences of BGL I were very similar to the putative β-glucosidase of *K. lactis* and β-glucosidase of *Kluyveromyces marxianus,* the peptide sequences of BGL II were similar to an unnamed protein of *Debaryomyces* (unpublished data). Both the enzymes have been placed in family 3 of the glycosyl hydrolase families. By way of cloning and expression, two more enzymes, namely Bgl I (Pandey and Mishra, 1997) and Bgl II (Sethi et al., 2002; Bhatia et al., 2005) were identified and studied. These multiple enzymes have been used for synthesis of various short chain cellooligosaccharides and other glycoconjugates. The synthesis of cellooligosaccharides (degree of polymerization 2–6) was demonstrated using Bgl I and Bgl II with glucose and cellobiose as the substrates. The separation of individual oligosaccharide is still a challenging problem in such synthetic reactions. We have reported some success in separation of these using a combination of charcoal adsorption and thin layer chromatography (Bacchawat et al., 2004). Transglycosylation reaction using *p*NPG as a donor and β-1-N-acetamido-D-glucopyranose, which is a glycosylasparagine mimic, as acceptor was also explored for synthesis of some special disaccharides. The yield of the reaction was 3% and both (1→3) and (1→6) linked disaccharides were synthesized using Bgl II (Kannan et al., 2004). The synthesized disaccharides β-D-Glc-(1→3)-β-D-Glc and β-D-Glc-(1→6)-β-D-Glc are important fragments of phytoalexin-elicitor oligosaccharides, which are involved in plant defense mechanisms. These oligosaccharides are regarded as essential players in the cellular communication between fungal pathogens and various plants (Geurtsen et al., 1999). In fact, the yeast cell wall contains 60% of these in their core structure. Many of these synthesized molecules may also serve to dislodge the pathogens from plants.

Another category of compounds, namely alkyl glucosides, are non-ionic surfactants which are useful for solubilization of membranes and for reconstitution of lipid vesicles (due to the amphoteric nature of the alkyl glucosides). The enzymatic synthesis of these molecules is of interest as the traditional chemical route involves a number of protection and de-protection steps. The use of toxic chemical catalysts

which remain at the end of the synthetic reactions is also not desirable. Very few microbial enzymes have been used for such synthesis and much of the work reported is with the almond β-glucosidase. We have reported synthesis of octyl-β-D-glucoside using BGL I (Wallecha and Mishra, 2003), BGL II (Wallecha, 2002) and Bgl II (Bacchawat et al., 2004). The molar yield of octyl glucoside was about 12% using BGL I in dimethyl sulfoxide stabilized single-phase reaction microenvironment. The yield of this compound was still increased to 40% (molar ratio with respect to glucose) by control of water activity in the system which favored the synthetic reactions over the hydrolytic ones (Mishra et al., 2007). The enzyme was also used successfully for driving the synthetic reactions with carbon chain length up to 12 to synthesize dodecanol glucosides.

The flavour compounds in various fruits are the various glucosides of terpene alcohols. There has been considerable interest in synthesis of these molecules using β-glucosidases. It is also expected that these will serve as a source of flavor precursors in fruit juices. We (Bacchawat et al., 2004) and others (Gunata et al., 1994) have reported synthesis of various monoterpene glucosides using β-glucosidase.

While the use of β-glucosidase for hydrolytic and synthetic reactions is widely accepted, a new finding could propel these enzymes into a different application. Recently, a new β-glucosidase-like activity has been reported from *P. etchellsii*. The purified enzyme of 50 kDa molecular mass was active on methyl-β-D-glucopyranoside (MUG), but very little activity was detected on *p*NPG, which is the most commonly used substrate for detection of β-glucosidase activity. Further, the gene sequence did not bear any significant sequence identity with either members of family1 or family 3 β-glucosidases (Roy et al., 2005). Apparently, the enzyme has a domain of hydrolytic activity similar to generic β-glucosidases but has some sequence similarity to GTP binding domains of other proteins (Mishra and Mishra, unpublished data). The sequence contained large stretches of Ser-Asp (SD), a feature shared with SD rich sequences of cell surface associated proteins from human pathogenic *Staphylococcus aureus* (McDevitt et al., 1994). The latter enzymes have been shown to bind to fibrinogen and assist in adhesion to human cells. Thus, the presence of SD repeat containing proteins in yeast cells is likely to throw new light on yeast cell surface properties and associated enzymes.

16.6 Conclusions

In this review, an overview of the yeast genetic material and its organization is presented. This has been largely possible due to the genome project. Out of nearly 6000 annotated genes, functional nature of only 40–45% is known. Some unique features of *S. cerevisiae* are lack of introns in the chromosomal genes whereas in the related *Sch. pombe* relatively large number of intron containing genes has been discovered. A close examination of the genomes could give features unique to Baker's yeast. Through an extensive collaborative project, single gene knockout mutants are commercially available. This is significant for two reasons (Astromoff

and Egerton, 1999): (i) it may allow to define the minimum genome size and (ii) the function of each of the putative genes can be elucidated. Although many of these genes may not have a specific 'phenotype' but their effect on the overall transcriptome and proteome can be analyzed in greater detail with the use of modern technologies such as DNA micro-array and Tandem mass spectrometry. These studies will also result in a better understanding of regulatory circuits operating in the cell. The vast amount of data has already propelled development of new computational methodologies.

The production of many valuable enzymes by different yeasts has been discussed. Since many of the yeasts are amenable to genetic manipulations, have a well-defined transformation system and have good vectors and hosts available, the enzymes can be produced in large scale.

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