YEASTS, MOLECULAR AND THERAPEUTIC APPLICATIONS

1. Introduction

The advent of recombinant DNA technology opened many exciting new uses for yeasts as it did for various other organisms. For the first time it became possible to precisely manipulate the genome of an organism and to introduce foreign genes that were capable of encoding a rare protein (see Fig. 1) or indeed of encoding a new pathway that could lead to the production of rare or even novel biochemicals. With the passing of time yeast have become the most popular eukaryotic microbe for such processes. The reasons for the popularity of yeast and some of the applications that yeast have acquired are discussed in this chapter.

A major omission from this chapter has been a discussion of genetically-modified yeasts for biofuel production. This is largely because efforts in this area have not progressed to the same extent as the other technologies presented. However, as the fossil fuel reserves decrease and become much more expensive, an increase in efforts to genetically-engineer yeast to utilize abundant carbon sources (such as cellulose) to produce biofuels will be seen. This will certainly now become a priority growth area for both private and public enterprise.

Fig. 1. Some traditional and new products from yeast. Some of the new products such as Hepatitis B vaccine and insulin are in widespread use and have saved the lives of millions of people.

2. Advantages of Yeast

2.1. Safety. Safety is probably the biggest reason for the choice of yeast for the manufacture of products from recombinant DNA technology. Experiences in the past decade have made us acutely aware that there can be real dangers and unacceptable risks in releasing recombinant organisms or their products for public consumption. Yeast technologies go a considerable way to addressing these concerns.

A major concern has included the effects of the release and spread of antibiotic, antimetabolic, herbicide or insecticide resistance genes into the environment where they can be transferred to other species. Expert committees comprising scientists, lawyers, ethicists and members of the public now regulate the environmental release of recombinant organisms after debate and consideration of the consequences. Concerns are real since considerable control is exerted over health with the use of drugs and chemicals to control diseases including infection and cell proliferation. The agricultural environment is also controlled with herbicides or insecticides. DNA techniques are available that readily allow production of yeast transformants that contain no antibiotic resistance genes: indeed yeast recombinants are usually selected to lack a nutritional requirement. Yeast fulfill the most stringent requirements possible, and recombinants that lack all foreign sequences can be produced. Such was the case for a modified yeast for bread making that was approved for use in the UK in 1990.

The second safety concern is what might contaminate the product. The public is now acutely aware of problems presented by mammalian systems and systems that depend on blood serum. Cells themselves or components of the culture medium may be contaminated by viruses, prions or endogenous retroviruses raising concerns that they or products derived from them could give rise to

disease. Yeast media are well characterized, can be safely made free of viral contamination by heat sterilization and can be produced totally by chemical synthesis if desired.

Yeast has a long track record of being used in the food and beverage industry with complete safety. Most of the population consumes yeast-derived products such as bread, cheese, chocolate, wine and beer from infancy to old age with no adverse effects. In fact even live yeast are not a problem, except for very small numbers of people who are severely immunocompromised. A small minority of the population, however, have yeast allergies which can render them susceptible to anaphylactic reactions to yeast-derived products. The allergenic compounds in yeast may include enolase and cell wall glycoproteins, giving hope that non-allergenic yeast could be engineered to produce yeast-derived products suited for the entire population (1). In the meantime those who suffer yeast allergies can escape harm simply by avoiding such products.

2.2. Large Scale Production. The production of yeast, including recombinant yeast is scalable from the laboratory to industrial production. This is well established in the beer and wine industries where fermentations of ten of thousands of liters are commonplace, with some breweries using fermentation vessels of 800,000 liters or more. Nontraditional yeast have now also been introduced into industrial processes with considerable success. For example the methylotrophic yeast *Pichia pastoris* was tested for the production of Hepatitis B vaccine, producing 9×10^6 doses from a 240 L fermentation (2).

2.3. Genetics and Molecular Biology. The yeast *Saccharomyces cerevisiae* has been studied for biological research since the studies of Louis Pasteur on fermentation in the 1860s. Its existence in the wild as a polyploid organism prevented genetic studies for years but successful haploidization in the Carlsberg laboratories, followed by its global distribution to researchers throughout the world, led to an intense development of yeast genetics. Thus *Saccharomyces cerevisiae* is often suggested to be the best understood organism. Its early use in biochemistry can be recognized by the isolation of the first enzyme, zymase, from yeast. The general name enzyme, given by Wilhelm Kuhne in 1878, comes from the Greek words *e*n and *zyme* which literally means *in yeast*.

The genetics of *Saccharomyces cerevisiae* continued strongly through the 20th century until molecular biology took over and reverse genetics developed. While the random isolation and study of mutants had led to much information about genes and their functions (genetics) those studies were always limited by the mutant phenotypes that could be found. Sequence analyses, on the other hand, led to the identification of numerous new open reading frames (ORFs) whose presence suggested a functional gene. Yeast molecular biology approaches allowed the deletion of these ORFs followed by analyses of the deletant strain to find a resulting phenotype (reverse genetics).

Saccharomyces cerevisiae was considered the model to decipher the genes in other eukaryotes so gene structure and function studies dominated much of the yeast research from 1980 onwards. By a huge manual effort that involved 74 laboratories the entire genome of *Saccharomyces cerevisiae* was unveiled in 1997 (3–11) the fruit of an intense international effort. It revealed approximately 6000 genes encoded by 12 million nucleotides, a gene for every 2 kb. This tight

packing of genes is not typical of higher eukaryotes whose genes are often interspersed with introns that become spliced out after transcription and do not appear in the final gene product. Thus human genes are an order of magnitude larger than yeast genes. Despite these differences the final gene products resulting from translation of human and yeast genes can be recognized on the basis of similarities and pattern recognition (12,13). In fact the identity of a human gene is often deduced from a comparison to the yeast gene where functions are usually known.

With the elucidation of the sequence of the yeast genome was the realization that there were many "open reading frames" that were capable of encoding products but there was no genetics to support any identification of function. This led to a new discipline "reverse genetics" where these ORFs were deleted so that a deleted function could be discovered. In practice, single gene deletants frequently led to the loss of a function (as determined by a battery of tests). However, in many cases no change can be determined in a deletant. One reason for this outcome is that two (or more) genes encode the same function: it is not until both genes are deleted that a function can be ascertained. In other cases the encoded function could be one that is not included in the regular battery of tests. For example, the phenotype conferred by gene within a yeast mitochondrial intron is to encode an endonuclease that directs propagation of the intron in crosses to strains that lack the intron (14).

While discussions in this section have so far focused on laboratory strains, the sequence of an industrial polyploid yeast has now been determined (15,16). This information paves the way for the exploitation of such a strain for recombinant DNA work and provides information on the history and relatedness of yeast species.

2.4. Yeast Transformation. The ability to genetically engineer yeast depends on being able to introduce, select for and stably maintain the introduced DNA sequences in yeast. This process is called transformation. The first reports of successful yeast transformation were in 1978 (17,18) where *leu2-* strains, lacking β-isopropylmalate dehydrogenase, were transformed into leucine prototrophs due to the uptake and expression of introduced *LEU2* genes (which encoded β-isopropylmalate dehydrogenase). Hinnen and co-workers (17) cloned the yeast *LEU2* gene onto a bacterial plasmid and introduced the chimeric plasmid into a *leu2-* yeast where it recombined into the chromosome. This recombination could be shown to occur through homologous crossover at the site of chromosomal *LEU2* gene, although in some transformants there was integration into the chromosome at other sites (nonhomologous recombination).

Jean Beggs (18) also used an *E. coli* plasmid containing the *LEU2* gene but her plasmid also contained sequences from a native yeast plasmid called the 2 micron circle plasmid (2μ plasmid). She found that her plasmid could also transform a *leu2-* yeast, but it was maintained as an episomal element and could be recovered from a yeast lysate and re-transformed into *E. coli*. It was the first *E. coli* – yeast shuttle vector.

Native Yeast Plasmids. The native 2 micron circle plasmid is found in most strains of *S. cerevisiae* and it forms the basis of most *E. coli* – yeast shuttle vectors which are the most popular plasmids for laboratory studies. The plasmid consists of two inverted repeats separated by a large unique (UL) and a small

unique (US) sequence. Within the unique sequences are just a few genes, including a recombinase (FLP) that enables recombination between the repeat sequences so that two forms of the 2 micron plasmid co-exist. The remaining genes, *REP1* and *REP2*, enable plasmid partitioning while *ori* 2μ serves as an origin of replication (19,20). Strains without the plasmid grow at a fairly similar rate. The plasmid achieves autonomous replication through a rolling circle mechanism and a crossover event so that two forms exist.

For the construction of vectors utilizing the 2μ plasmid the entire plasmid can be recombined with an *E. coli* vector or just the 2μ ori sequence. Of the many known species of yeast, few yeast are known to have DNA plasmids and in each case they are functionally identical to *Saccharomyces cerevisiae* 2μ plasmids. The plasmids include pSR1, pSB3 and pSB4 from *Zygosaccharomyces rouxii* (formerly classified as *Zygosaccharomyces bailii*), pSM1 from *Zygosaccharomyces fermentati* and an unnamed plasmid from *Pichia membranaefaciens* (21,22). Interestingly the 2μ plasmids have species specificity, although the one found in *Kluyveromyces drosophilarum* has been manipulated for use in its industrial equivalent *Kluyveromyces lactis* (which does not normally have a plasmid) (23).

Selectable Markers. Another plasmid found in yeast is a double-stranded RNA plasmid called killer plasmid, because it encodes a toxin that inhibits other yeast (24). It is, of course, difficult to manipulate and has had very little use in comparison to the shuttle vectors.

Further developments on shuttle vectors have involved the considerations of the selection of genetically-engineered yeast. The development of selection systems for many genetically-engineered organisms has focused on selection of a dominant marker such as antibiotic resistance, but the use of such markers is now considered less desirable (as discussed under Safety).

In yeast two dominant markers have persisted. The KAN^R gene is a selectable maker that can be used in *E. coli* where it confers kanamycin resistance and in yeast where it can lead to G418 (Geneticin) resistance (25). The use of a single selectable marker for yeast and *E. coli* has the advantage of reducing the size and complexity of a shuttle vector. The KAN^R gene has also been used extensively in construction of the yeast deletion library (discussed below). *CUP1* is another notable dominant marker that has been used for industrial strains that are usually polyploid prototrophs, making them unsuitable for accepting auxotrophic markers. *CUP1* encodes copper metallothionein and can confer copper resistance to strains in a manner that is dependent on copy number.

The plethora of auxotrophic mutations available in yeast has led to numerous markers being introduced onto plasmids for selection in yeast. Such markers, in addition to *LEU2* (discussed previously), include *URA3, HIS3* and *TRP1*, which encode, orotidine-5′-phosphate decarboxylase, imidazoleglycerol-phosphate dehydratase, and phosphoribosyl anthranilate isomerase. A principle of best practice that has emerged over the past few years is that genetically-engineered yeast should have minimal foreign DNA sequences. This means that vectors that can be shuttled backwards and forwards between yeast and *E. coli* through their use of selectable markers and origins of replication that work both for both bacteria and yeast are unacceptable.

For industrial use, shuttle vectors often tend to be replaced by integration strategies to provide stability. In addition, industrial yeasts may be protrotrophs

and have no capacity for selection or replication of autonomously replicating plasmid sequences. The approaches to producing recombinants this way include homologous integration where gene replacements and manipulations can be made, such that the foreign sequence encoding the protein of interest might be the only foreign sequence. This can involve production of a linear DNA sequence with ends that are homologous to the yeast chromosome. Such ends are highly recombinogenic and will drive a crossover event.

Another feature that one may look for in yeast plasmids is low copy number, which may be important for complementation studies. Centromeres (or *CEN* sequences), the essential elements that link chromosome arm and maintain mitotic and meiotic stability, can be introduced into yeast plasmids to maintain the plasmid at one (or two) copies per yeast cell. Such plasmids require a weak autonomous replication sequence (ARS) element that can be obtained from the yeast chromosome: they are incompatible with 2μ ori sequences that drive a high copy number.

There are also criteria to obtain high plasmid number such as use of the *LEU2d* selectable marker. This selectable marker lacks most of the regular promoter: there are only 30 nucleotides of *LEU2* sequence upstream of the start codon. With *leu2-* cells carrying a plasmid with a *LEU2d* selectable marker, the vector copy number is driven to higher levels in the absence of leucine so that the transformant has enough β-isopropylmalate dehydrogenase (the *LEU2* gene product) to provide the required amount of leucine. An example of a vector with excellent attributes for production of foreign proteins in yeast is pYEULBX (sold commercially as pYEX-BX; Fig. 2) (26).

Yeast Artificial Chromosomes. Finally, another class of yeast vectors are Yeast Artificial Chromosomes (YACs). YACs are useful for cloning very large pieces (up to 1 megabase) of DNA, which has made them useful for sequencing and gene mapping projects like the Human Genome Project. Also they are used for the assembly of novel biosynthetic pathways in yeast. In other words, a number of genes may be assembled into a YAC such that an entire novel pathway may be expressed in yeast. The starting materials for YAC cloning are large insert pieces of DNA and a YAC vector which is composed of an *E. coli* plasmid that can be cut with restriction endonucleases to liberate two mini-chromosome arms, each with a telomere end and having a different selectable marker. In addition one of these mini-chromosome has a CEN sequence.

2.5. Secretion. Another attribute of yeast is the ability to be able to secrete proteins. The multicellular fungi probably are among the best organisms at high level secretion but the yeasts can secrete proteins at reasonable level too and their industrial acceptance makes them good hosts for foreign protein secretion. Some proteins that have been found suited for secretion are human serum albumin and antibodies. These will be discussed separately. *Saccharomyces cerevisiae* has GRAS (generally regarded as safe) status from the FDA, making it appear ideal for therapeutic protein production. It does not have the extensive record for secretion of foreign protein (possibly due to a lack of commercially available expression systems for *S. cerevisiae*), as a considerable number of alternative yeast. Yeast with good track records in protein secretion include *Pichia pastoris*, *Hansenula polymorpha*, *Kluveromyces lactis*, and *Candida utilis*.

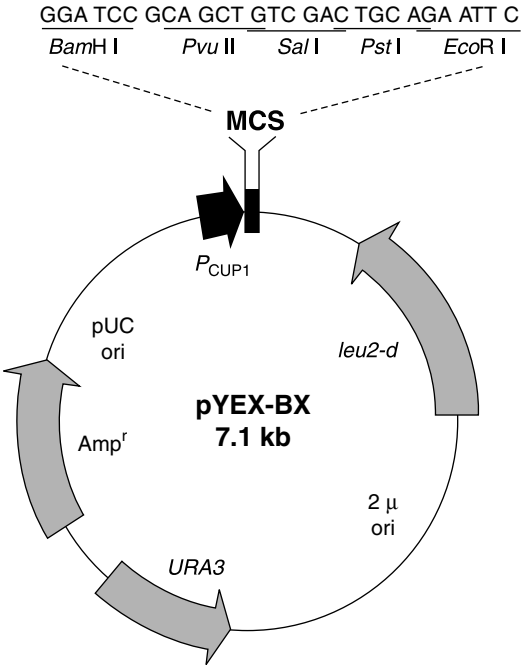

Fig. 2. Yeast expression vector pYEX-BX. This is a yeast – *E. coli* shuttle vector designed for the copper-inducible production of foreign proteins in yeast. Foreign genes can be cloned into the multiple cloning site (MCS) which contains five unique restriction sites. Following ligation plasmids are selected and amplified in *E. coli* as ampicillin resistant transformants. Recombinant plasmids are recovered from *E. coli* and then transformed into a *leu2 ura3* strain, selecting for transformants that do not require uracil. For expression of the foreign gene transformants are grown in the absence of leucine also to amplify the plasmid further. The *leu2d* selection marker comprises the *LEU2* with just 30 nucleotides of upstream sequence, which makes for a poor promoter. In order to survive in the absence of leucine the pYEX-BX plasmid copy number becomes highly elevated to provide enough transcripts for *LEU2* gene product that is required.

3. New High Value Protein Products from Yeast

Some of the applications of yeast using recombinant DNA technology are given in Table 1. The examples are not exhaustive but are given to illustrate the diversity of applications and to perhaps give the reader some sense of future possibilities.

3.1. Human Insulin. One of the biggest medical successes from recombinant yeast technology has been the production of human insulin, since 1986 (29). Since the demand for insulin was going to exceed supply in the 1980s, the use of recombinant DNA technology was both timely and lifesaving for diabetics. In addition, the yeast-derived product more closely resembled human insulin than porcine insulin, providing increased efficacy and decreased side effects. Novo is the world's largest supplier though yields are not usually publicized. However, a Chinese group reports secretion levels of 1.5 g per liter from high density fermentation of *Pichia pastoris* (30).

Table 1. **Purified Proteins Available from GM Yeast**[a]

Protein	Commercial product name	Manufacturer	Expression system
Hepatitis B surface antigen	Ambirix	GlaxoSmithKline	*S. cerevisiae*
	HBVAXPRO	Aventis Pharma	
	Hexavac	Aventis Pasteur	
	Infanrix-Penta	GlaxoSmithKline	
	Pediarix	GlaxoSmithKline	
	Procomvax	Aventis Pasteur	
	Twinrix	GlaxoSmithKline	
	Hepavax-Gene	Rhein-Biotech	*H. polymorpha*
human papilloma virus antigen	Gardasil	Merck	*P. pastoris*
insulin	Actrapid	NovoNordisk	*S. cerevisiae*
	Novolog	NovoNordisk	
	Insugen	Biocon	*P. pastoris*
urate oxidase	Elitex	Sanofi-Synthelabo	*S. cerevisiae*
Glucagon	Glucagen	NovoNordisk	*S. cerevisiae*
granulocyte macrophage colony stimulating factor	Leukine	Berlex	*S. cerevisiae*
hirudin/lepirudin	Refuldan	Hoechst	*S. cerevisiae*
platelet-derived growth factor	Regranex rh	Ortho-McNeil Pharma (U.S.), Janssen-Cilag (EU)	*S. cerevisiae*
hirudin/desirudin	Revasc	Aventis	*S. cerevisiae*
PDFG-agonist		ZymoGenetics	*S. cerevisiae*
human serum albumin	Recombumin	Delta Novozymes	*S. cerevisiae*
transferrin	DeltaFerrin	Delta Novozymes	*S. cerevisiae*

[a]From Refs. 27,28.

3.2. GM-CSF. About the same time granulocyte macrophage colony stimulating factor (rhu GM-CSF) was another protein that was found to be successfully produced in yeast. The amino acid sequence of the recombinant protein differs from the natural human GM-CSF by a substitution of leucine at position 23 (31). The yeast derived product was found to be of particular use in helping neutrophil recovery in older individuals recovering from acute myelogenous leukemia (AML), after bone marrow transplantation, and before and/or after peripheral blood stem cell transplantation. It also raises CD4 in HIV patients (32–34). Sargramostim (35) has been selected as the proper name for yeast-derived rhu GM-CSF, although leukine is alternative name.

3.3. Human Serum Albumin. Serum albumin is the major protein in blood with plasma levels of over 40 g/L. In addition serum albumin has properties that make it an important agent in pharmaceutical delivery. Although large amounts can be recovered from blood, the risks associated with transmission of viruses and prions have forced a need to look for recombinant sources. Yeast have proven to be very efficient in producing human serum albumin (HSA). Scientists Novozymes at Delta reported successful secretion of HSA from *Saccharomyces cerevisiae* in 1990 (36), showing considerable dependence on a

secretion leader sequence. They tried a total of five leader sequences, including those of *S. cerevisiae* alpha factor, the natural human serum albumin, and *Kluyveromyces lactis* killer toxin, all of which could be cleaved by the yeast KEX2 protease (37). Shortly after, *Kluyveromyces lactis* was found to be an excellent yeast capable of secreting some grams of HSA for each litre of culture (38,39).

The FDA approved Novozymes Delta's recombinant HSA in 2005. Their *S. cerevisiae* derived product, "Recombumin" is marketed as a prion-free version of HSA. Its manufacture in yeast is free of any animal or human-derived products making it safe for vaccines, cell culture, and drug delivery.

3.4. Human Transferrin. Human transferrin (DeltaFerrin) has also been produced in *Saccharomyces cerevisiae* by Novozymes Delta. The product is free of viruses and transmissible spongiform encephalopathies and superior at delivery of iron to cells in culture when compared with traditional culture media (40).

3.5. Human Antibodies. Therapeutic humanized monoclonal antibodies for the treatment of diseases such as non-Hodgkin's lymphoma and Her2-associated breast cancer, are now available but still very expensive. It is possible that yeast may perform this task at some stage in the future due to successes in producing antibodies, both full length (Fab) and single chain antibodies (scFv), in yeast. Antibody production in yeast has been reviewed by Joosten and colleagues (41) who consider that inexpensive antibody production, will enable their use in very broad applications in the future. The U.S. company GlycoFi, recently produced humanized IgGs in glycoengineered strains of *Pichia pastoris* (42) and the performance of these of these antibodies appears very promising (43). Recognizing the value of this technology for the production of a new range of human therapeutics, included human antibodies, Merck acquired GlycoFi for $\$400 \times 10^6$ in 2006, the largest acquisition of a private company in U.S. history. It is also noteworthy that the GRAS status of *S. cerevisiae* and the ease of its use offers some competitive advantages that companies such as ApoLife appear keen to exploit.

4. Vaccines from Yeast

Yeasts have an excellent track record for the production of vaccines. The vaccination process involves the stimulation of the host immune system with an antigen that appears equivalent to the native protein. The antigen could be a viral protein, a bacterial protein, a protein from a eukaryotic pathogen, such as the malaria parasite, *Plasmodium falciparum*, or even a protein from a parasitic worm. Having identified a host protective antigen (a protein that elicits immunity to infection) the major requirement is to produce that antigen in its correct form. Traditionally, vaccines have been produced as heat-killed organisms or live attenuated organisms. In the latter case the organism may have been attenuated in its virulence by extensive passaging. However, such practices may be expensive and present safety concerns. Thus there have been attempts to produce such proteins by cloning the genes that encode them for their expression in a safe microbial host such as yeast. A considerable number of "subunit vaccines" produced in yeast are listed in Table 2.

Table 2. **Vaccine Proteins Produced in GM Yeast**[a]

Protein	Expressed in:	Reference
human papilloma virus antigens	*P. pastoris*	See patent literature
infectious bursal disease virus VP2	*S. cerevisiae*	44
	P. pastoris	1,45
Newcastle Disease Virus	*P. pastoris*	37
Plasmodium falciparum Pfs 25	*P. pastoris*	46
Plasmodium falciparum AMA1	*P. pastoris*	46
influenza neuraminidase	*P. pastoris*	47
influenza hemagglutinin	*P. pastoris*	48
Bacillus anthracis protective antigen	*S. cerevisiae*	20
Botulinum neurotoxin type F	*P. pastoris*	49
Schistosoma japonicum paramyosin	*P. pastoris*	19
hookworm secreted protein ASP-1 and ASP-2	*P. pastoris*	19
cattle tick Gavac	*P. pastoris*	50
tumor and HIV antigens	*S. cerevisiae*	51

[a]Does not include hepatitis B vaccines.

4.1. Hepatitis B Vaccine. The first and another of the biggest medical successes from recombinant yeast technology has been the production of Hepatitis B vaccine. A landmark achievement was the discovery that Hepatitis B surface antigen could be expressed in yeast and that it resembled the 22 nm particles that are seen in the blood of chronic carriers (52,53). Such particles could be readily purified from yeast leading to their formulations with adjuvants to produce vaccines (52). Although the Hepatitis B vaccine was originally developed as a relatively expensive vaccine for profession health care workers, its efficacy and utility was noticed by the World Health Organization (WHO). The WHO were instrumental in ensuring that the vaccine would be available to a much wider community at a fraction of the original market price of $100. Thus entire communities of some countries were vaccinated resulting in widespread elimination of Hepatitis B. Because Hepatitis B is often a cause of liver cancer, the vaccine has often been hailed as a leading cancer prevention vaccine too.

The development of yeast vectors and yeast transformation technology in the late 1970s paved the way for the early success of Hepatitis B surface antigen (HBsAg) expression in *Saccharomyces cerevisiae*. *S. cerevisiae* has been so suited to the Hepatitis B vaccine that its use continues today. However, other yeast are capable of the same production and have been employed to produce the vaccine (54–57). Such yeast include *Pichia pastoris* which has been used in India for some time now to produce a Hepatitis B vaccine known as Shanvac (46).

4.2. IBDV Vaccine. Success with the Hepatitis B vaccine encouraged the search for other viral subunit vaccines. The second such vaccine was the Infectious Bursal Disease Virus (IBDV) vaccine (44). IBDV infects the bursa of Fabricius in chickens. The bursa of Fabricius produces the B cells that affect immunity, and IBDV infection results in birds with immune deficiency. The traditional IBDV vaccine was produced from infected bursa making it one of the most expensive poultry vaccines. However, protection against IBDV could also be achieved by immunization with the IBDV protein called VP2.

The biggest challenge was to produce the VP2 subunit at cents per dose to make it a cost effective for the poultry farmers. Both *Escherichia coli* and *Saccharomyces cerevisiae* were employed for production and testing of VP2. Both hosts produced VP2 that reacted with viral neutralizing antibodies and was highly immunogenic. Indeed the VP2s from both sources appeared indistinguishable. However, in vaccinated chickens the antibodies raised from *Saccharomyces cerevisiae* VP2 were protective (44) while those from *Escherichia coli* VP2 were not protective. Since post translational modifications did not account for the difference, the result suggests that VP2 only takes on a correct protein conformation when the antigen is produced in the yeast.

Improvements to the vaccine were achieved using *Pichia pastoris* and 250×10^6 birds were reported as being vaccinated in 2003 (1).

4.3. Other Vaccines. Other examples in the literature of yeast producing successful vaccines include one for malaria (31). The antigen was merozoite surface protein-1 and it provided complete protection to mice against challenge with *Plasmodium yoelii*. It would appear that the technology could equally be successful in humans. *Bacillus anthracis*, which causes anthrax, is another example of an organism that can be suppressed by a yeast-derived vaccine (19).

The methylotrophic yeast, *Pichia pastoris*, has the largest track record for vaccine proteins. The highly A + T rich sequences of *Plasmodium* present major problems for expression in *Saccharomyces cerevisiae*, however, *Pichia pastoris* appears to handle such sequences more favorably (58). A large number of *Plasmodium* proteins have now been produced in *Pichia* for evaluation as vaccines (46,58–61). Other vaccines being developed in *Pichia* include those listed in Table 2 (62).

4.4. Whole Yeast Vaccines and Antigen Display on Yeast. While the production of highly purified antigens as biological agents has significant uses, there are also uses for whole yeast that have a foreign antigen displayed on their cell surface. This can have advantages in that there is no complex purification involved and that the antigen is displayed on a convenient and safe carrier. Stubbs and colleagues have demonstrated that yeast with recombinant antigens on their surface can elicit protective cell-mediated immunity (63). Similarly, when whole yeast expressing *Actinobacillus pleuropneumoniae* ApxIIA were fed to mice protection against *Actinobacillus pleuropneumoniae* could be achieved (64). This technology of antigen display also has numerous nonvaccine applications.

5. Yeast as an Enabler

5.1. Two Hybrid Screening Applications. Yeast has become important in the drug discovery process because of special molecular developments. One such development is the two hybrid screening approach (20). This allows the *in vivo* detection of protein-protein interactions. The system, outlined in Figure 3, relies on the discovery that the GAL4 transcription factor can be broken apart into separate domains, a DNA binding domain (BD) and a transcription activation domain (AD). But when these domains are brought together again GAL4 function can be restored. An elegant way in which the domains can be

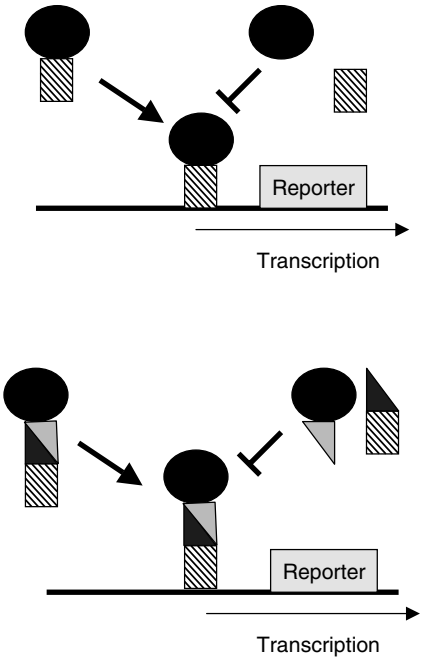

Fig. 3. The yeast two hybrid system. The GAL4 transcription factor has two domains: a DNA binding domain (BD, represented as rectangles) and an activation domain (AD, represented as circles). It activates transcription upon binding to an upstream activation sequence (UAS). For two hybrid assays, a specially constructed strain with the GAL4 UAS upstream of a reporter gene is used. If the BD and AD are separated there is no transcription. However, if they can be brought together transcription is activated. Consider the case of two hybrid proteins that are made by directing expression of the BD fused to protein X (represented as the blue triangle) and the AD protein fused to protein Y (represented as the green triangle). If the X and Y interact they reconstitute a GAL4 transcription factor which directs expression of the reporter. If they do not interact there is no activation of the reporter. See online version for color.

brought together is to add interacting proteins onto AD and BD. Their interactions then brings AD and BD together to restore transcription activation. A further refinement is to include a reporter gene so that GAL4 activation can be readily detected. A reporter can include the bacterial β-galactosidase gene (*lacZ*) reporter whose activation can be detected by a chromogenic color assay. Another assay includes the production of an enzyme like the *HIS3* product that is lacking in the *his3-* host strain. Such a procedure provides an approach not only to screen for an interaction but to detect an interaction at a very low frequency, even one in 10 million cells or more. Such interactants can be readily selected from the mass of non-interactants and provide an approach to screen a large library of constructs.

Therefore, not only can protein interactions be finely examined on a pairwise basis, the interaction domains can be studied. However, one of the most exciting options is library screening where one can ask what does my protein of interest interact with in the proteome? Thus one might start with the protein

of interest fused to the AD domain and screen against a library of cDNAs fused to the BD domain. Putative interactions can be detected as described above and the precise identification of the interacting protein can be made by recovering the library plasmid by back transformation or by PCR sequencing.

Other variations of the two hybrid system include reverse two hybrid screening which can be employed when looking for compounds that disrupt a protein–protein interaction. Likewise, a one hybrid screening can be used to find transcription factors or the two hybrid system can be used to find compounds that disrupt transcription factor binding.

5.2. Gene Arrays. Another advance that has been made throughout biology is the use of gene arrays. Gene arrays allow a genome-wide expression analysis of yeast. The arrays have opened new insights into pathways and gene regulation, helping to decipher unexpected ways in which yeast respond to stimuli. A huge amount of data now exists and much of it is publicly available for perusal. Such insights are particularly useful for yeast biotechnology applications but they also impact on many other studies of cell biology, including studies on human diseases.

Gene arrays comprise sequence probes, often as long oligonucleotides, that are immobilized on a membrane or a glass slide. The predominant formats are now glass slides that contain representations of all 6000 yeast genes. Other variations can include representations of multiple regions within genes and of intergenic regions too. Of course, the probes are printed in duplicate and appropriate controls are included on the slide. Commonly, the arrays are used to compare expression profiles of two yeast grown under different conditions, or of a mutant strain and its wild-type parent, or of a transformant bearing a human "disease protein" with a transformant that lacks that protein. The question the gene array answers is, what are the differences in the gene expression profile resulting from the genetic or environmental change. In the procedure, RNAs are prepared from the comparison strains and then they are fluorescently labeled, one with a red fluorescent dye and the other with a green fluorescent dye. Equal amounts of the different RNAs are then hybridised to a gene array slide. The amounts of red and green fluorescence are then measured by a sophisticated scanner that can provide a high resolution picture (Fig. 4) and measurements of the amounts of red and green fluorescence on each spot. The majority of genes will have unaltered expression that will correspond to equal amounts of green and red fluorescence. However, altered expression leads to differences in the ratio of red to green fluorescence. This can be discerned by visual inspection of the slides, using the picture captured by the scanner, or from ratios measured by software such GenePix and GeneSpring.

The first gene arrays reported on altered expression of yeast genes under altered culture conditions (65). The power of the technique was soon adapted for human genes where it was shown to be useful to study the differential expression of genes in different types of cancer cells, aiding diagnosis of the cancer cell type (66). In addition, arrays can also predict the drug sensitivity of various cancer cells (67).

5.3. Yeast Gene Deletion Libraries. Another powerful technique in yeast has been the construction of collections of thousands of yeast gene deletants (68). The strategy for construction of gene deletants is shown in

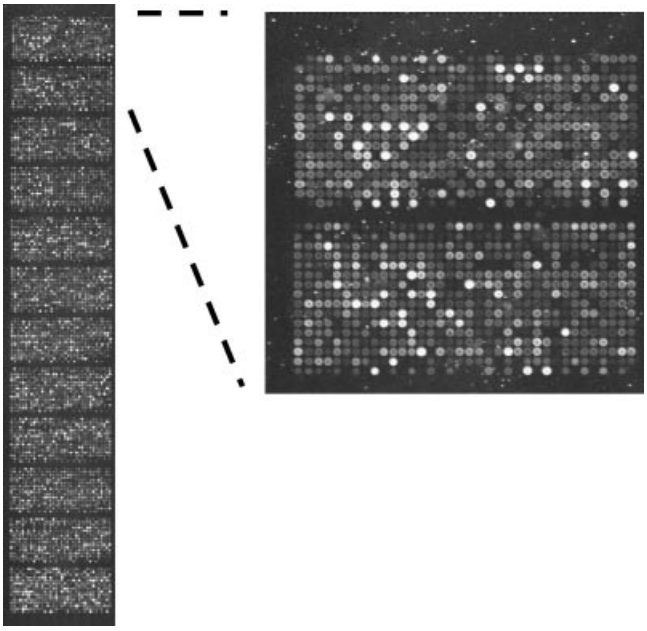

Fig. 4. Yeast gene arrays. An entire slide is shown, along with the top two sectors under magnification. Red and green spots indicate genes that are up or down regulated. See on-line version for color.

Fig. 5. It relies on causing a chromosomal replacement of each and every ORF with a selectable marker (a KAN^R gene that confers G418 resistance). The linear DNA segment to drive this integration comprises ends that are homologous to around 50 nt of sequence upstream and downstream of the gene to be disrupted with the *KAN*^{R} gene in between the upstream and downstream yeast ORF sequences. This is prepared by using long primers to PCR amplify the *KAN*^{R} gene, with the primers already containing the upstream and downstream yeast ORF sequences.

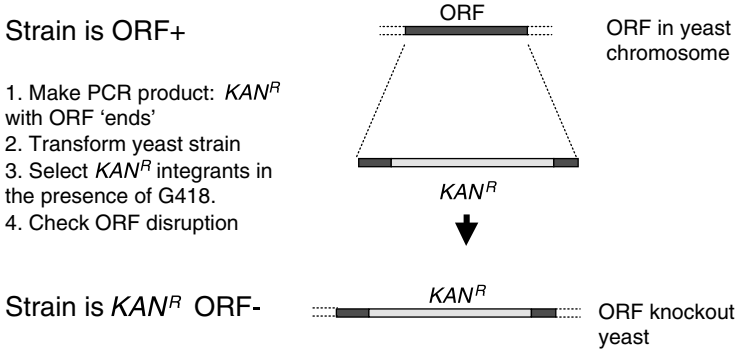

Fig. 5. Yeast gene deletion strategy.

The gene deletants include every gene or ORF and they were derived from isogenic parental strains comprising haploid strains of both mating types, plus homozygous and heterozygous diploid strains. Because some gene deletions cause a loss of viability the collection lacks such strains from the haploid and homozygous diploid series. However, they can be accessed in the heterozygous diploid series.

For many years, yeast mutants have been employed to unravel gene functions. The total or conditional loss of a function due a gene mutation is a powerful approach that has helped yeast genetics to lead the field in gene identification. When a similar gene was found in another organism, its function could be deduced based on its sequence similarity with the yeast, and in some cases complementation could even be achieved, leading to the elucidation of functions in other organisms at a rapid rate. Of course the above strategy is limited by the mutants one can obtain. This is exacerbated by the problem that the selection of mutants with a phenotype is an integral step.

A way around these issues is reverse genetics, through molecular biology. This involves the introduction of mutations into a gene and the observation of (or the search for) a phenotype. In many cases a new phenotype is not observed. Gene duplications can be the reason. If a gene duplication is suspected then both genes may need to be deleted to observe a phenotype. Of course another scenario is a phenotype that is not readily apparent, possibly a novel phenotype not anticipated in yeast. There are still many yeast ORFs that have no function ascribed to them.

6. Biotechnology

6.1. Gene Manipulations on Traditional Yeast Biotechnology. Recombinant yeast offers amazing new opportunities for the food, wine and brewing industries. Research has been widespread across the industries and has included engineering to remove the starch haze from beer, the re-design of wine yeast to degrade cellulose and modification to improve dough rising in bread making. Much of the research that is performed in this area is highly commercial and does not often appear in scientific literature. If published at all, it appears in patents, or when approval is sought for introduction into a process that involves the public.

Bread Making. In 1990, a modified yeast became the first GMO to be approved anywhere in the world for use in food (69). Approval was given to the yeast manufacturer Gist-Brocades to use this yeast in bread in the UK. Compared to traditional yeast, it produced around 30% more carbon dioxide during dough fermentation. Many other bread making improvements have been made to yeast but most of the advances are in the patent literature.

Cheese Manufacture. Cheese manufacture requires a prochymosin which is normally derived from calf stomach. However, it was found to be efficiently made in recombinant yeast as early as 1983 (70,71). The ability of *Kluyveromyces lactis* to secrete prochymosin at high levels has led to it being one of the hosts of choice (70). The recombinant DNA prochymosin is now marketed as Rennet Maxiren by Gist-Brocades N.V. (Netherlands).

Brewing. Beer made by recombinant yeast has also been approved for sale. The first example used a GM brewer's yeast that could break down dextrin to glucose which could then be fermented to ethanol. The beer produced contained fewer calories and about 1% more alcohol than beer made with unmodified yeast. It was produced by the Brewing Research Foundation International.

The Wine Industry. In 2003 a GM wine yeast (strain ML01) received GRAS (generally recognized as safe) recognition from the United States Food and Drug Administration (FDA), which allowed it to be used in wine production (72,73). The strain included a gene for a malolactic enzyme from the bacterium *Oenococcus oeni* and a malate permease gene from the fission yeast *Schizosaccharomyces pombe*. The advantage of this strain is that it converts malic acid to lactic acid and reduces the acidity of the wine. It also spares the normal requirement for lactic acid bacteria to carry out the malolactic acid fermentation. The foreign genes were integrated into the chromosome with transformants being selected as phleomycin-resistant co-transformants. Phleomycin resistance was carried on a plasmid lacking chromosomal yeast sequences. Thus it was unable to integrate and could be spontaneously shed. Of the original phleomycin-resistant co-transformants, integrants were searched for on the basis of ability to produce lactic acid. The company distributing the strain is the Springer Oenologie, Lesaffre Group, of North America. Their advertising reports improved wine qualities including softening of the wine's mouth feel by decreasing its acidity and reduced buttery flavours (diactyl) due to lactic acid secondary metabolism.

6.2. New Biosynthetic Pathways in Yeast. The abilities of multiple foreign genes yeast to be inserted into yeast, through the use of multiple plasmids but more preferentially through chromosomal integration or the construction of a YAC, means that a new pathway can be introduced into yeast. Two examples given below are the production of a mammalian hormone precursor hydrocortisone and a valuable plant-derived compound like artemisinin.

Hydrocortisone Production. Hydrocortisone is the major steroid of mammals and is the preferred starting material for the synthesis of a variety of drugs including those with antiproliferate and antiinflammatory effects. Its first reported chemical synthesis in 1952 required around 40 steps, however, the production in *Saccharomyces cerevisiae* (74) was achieved by addition of a biosynthetic pathway from mammals. The production of the pathway in yeast required the introduction of 13 engineered genes. This appears to be the most complex engineering reported in yeast so far.

Artemisinin Production. In 2005, artemisinin became the drug of choice for malaria due to widespread resistance to other drugs like antifolates and chloroquine. However, the cost of production of artemisinin and the inability to meet demand causes major problems. If the entire available land in the world were put to producing *Artemisia annua*, the plant from which artemisinin is derived, there would still be insufficient artemisinin to meet needs. In addition production costs of around $2.40 per treatment are still beyond the reach of health budgets for some African countries.

Alternative sources for artemisinin that have been examined include chemical synthesis and GM plant culture, however, these have not provided advantages over the traditional source. Production in yeast has offered a solution. The expression of three plant genes in *S. cerevisiae* has enabled production

of the artemisinin precursor, artemisinic acid, at levels of 100 mg per liter (75). It is estimated that this will bring production costs to 10% of the current cost.

7. Yeast in Medical Research

7.1. Infectious Diseases. The safe and convenient handling of GRAS yeast means that there have been many successful attempts to employ yeast as a model for various infectious diseases. The entire gamut of infectious disease has been considered but impacts have been greatest for pathogenic agents that are difficult to handle. Some examples include prion disease, viral disease, and malaria.

Fungal Diseases. Some yeast (notably *Candida* and *Cryptococcus* species) cause disease and there is important research that focuses on the control or prevention of infections. Much of this research focuses on drug targets that differentiate yeast growth from the hosts on which they live. For example, ergosterol synthesis is frequently targeted since it is an essential component of yeast (and fungal) membranes; in contrast, mammals employ cholesterol in place of ergosterol.

A fungal pathogen of particular interest is *Pneumocystis carinii* (now named *Pneumocystis jirovecii*) that causes *Pneumocystis carinii* pneumonia (PCP). It deserves special mention because it exemplifies the opportunities and reasons for wanting to use yeast as a model. *Pneumocystis jirovecii* is a major pathogen for the immunocomprised but can be treated with sulfa drugs that target folate synthesis, another pathway that is unique to microbes and plants (Fig. 6). The problem was that sulfa drug resistance occurred readily with

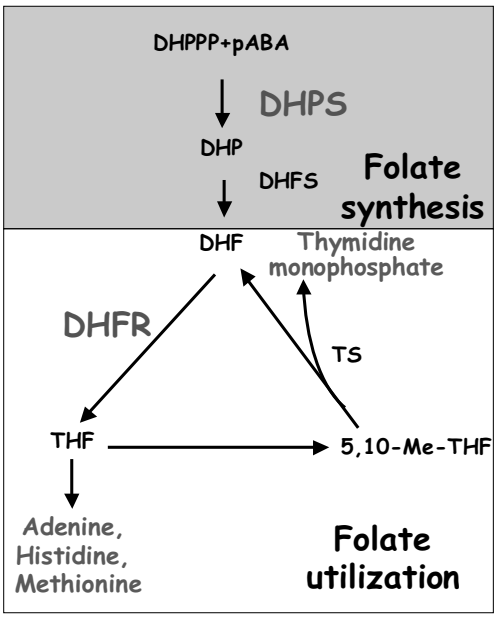

Fig. 6. Folate synthesis and utilization.

changes in the folate synthesis enzyme dihydropteroate synthase (DHPS) being associated with the resistance (76,77). Although the DHPS changes were the suspected cause of the resistance, the lack of a *Pneumocystis jirovecii* culture system outside the human lung meant it was difficult to establish this. However, similarities between the *P. jirovecii* and *S. cerevisiae* DHPS led to the changes being modeled in the yeast DHPS where they were found to lead to sulfa drug resistance (78). Observations that this occurred in a stepwise process provides further insights into the development of drug resistance (78).

Malaria. One of the world's biggest infectious diseases is malaria, a disease that kills a child every 30 seconds. Again the emergence of drug resistance has led to major problems. Since the world wide spread of chloroquine resistance, antifolate combinations have become first line drugs in many countries. Antifolate combinations comprise a DHPS inhibitor and an inhibitor of dihyrofolate reductase (DHFR), an enzyme that is involved in utilisation of folate (Fig. 7). Although very efficient and cost effective for developing countries, resistance has emerged. Changes in both DHPS and DHFR have been associated with resistance to antifolates.

Malaria is caused by one of four *Plasmodium* species with *Plasmodium falciparum* being the most dangerous. Because *Plasmodium* is an obligate parasite of red blood cells and requires culture in serum, the testing of drug resistance is particularly difficult, particularly with the complexities of endogenous folate in such media and cells. The complexities can be reduced by studying the effects of antifolate drugs in yeast which can be grown in folate-free media. Such studies have been eloquently performed by Sibley and colleagues where wild-type and mutant *Plasmodium falciparum* DHFR enzymes have been expressed in yeast in the place of yeast DHFR. This has allowed the evaluation

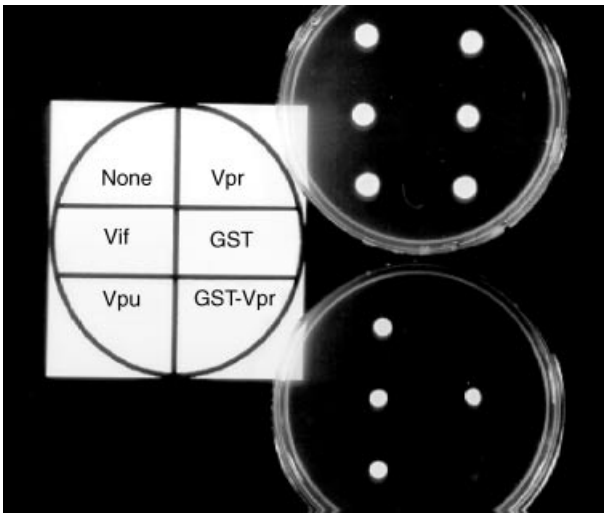

Fig. 7. Expression of the auxiliary proteins, Vif, Vpu and Vpr, from the HIV-1 virus. The top panel shows growth of transformants without induction while the bottom plate shows growth in the presence of the inducer, copper sulfate. The protein Vpr (and Vpr fused to glutathione-*S* transferase (GST)) does not allow growth.

of changes with respect to resistance to DHFR inhibitors (79), as well as the investigation of novel inhibitors.

Viral Disease. Viruses exert their effects through the expression of their genes inside particular host cells. Thus it can be relevant to consider the expression of individual viral genes in yeast to ascertain their effects on the cell which in turn can help with preventative strategies.

As an example consider the curious protein, Vpr, a HIV-1 protein now known to be composed of 96 amino acid residues. Early work on the isolation and culture of HIV-1 in the laboratory led to the selection of HIV-1 isolates that grew well in culture; however, they had truncated Vpr proteins due to mutation in Vpr-encoding sequences. In contrast, the sequence analysis of HIV-1 directly isolated from peripheral blood without passage showed that Vpr in an infected person always encoded the longer protein of 96 amino acid residues.

The expression of HIV-1 Vpr in *Saccharomyces cerevisiae* led to the discovery that normal Vpr induces a growth arrest (31) (Fig. 7). Further, it was shown that the region of Vpr that caused the growth arrest was near the C-terminus of the peptide. A flurry of studies in lymphocytes (26,80,81) validated the yeast findings and further suggested that the growth arrest associated with Vpr enabled HIV-1 to achieve increased virus production.

Prion Disease. Prions are infectious proteins that can lead to disease in humans. They appear to derive from normal cellular proteins that undergo a structural change into a highly stable beta-sheet form that is resistant to the action of heat and proteases. Their insidious nature is due to their being able to "transform" the normal cellular protein to the prion state, hence their infectivity. Human and important animal diseases caused by prions include the transmissible spongiform encephalopathies, mad-cow disease, Creutzfeldt-Jakob disease, chronic wasting disease, scrapie, Gerstmann-Straussler-Scheinker, syndrome fatal familial insomnia, and kuru.

Two prions, psi+ and ure3, have been studied for a number of years in yeast (46,76,82). In initial studies, they were a curiosity because of their cytoplasmic inheritance. However, over the past decade they have provided much insight into mammalian prion diseases. Of particular relevance is the genomic response to the presence of prions and the ability to "cure" prions from yeast (20).

7.2. Cellular Diseases. Many cellular processes exist in both yeast and human cells so it is not surprising that many cellular diseases can be studied in yeast. For example, yeast studies have had major applications to cancer research. The 2001 Nobel Prize was awarded to yeast geneticists, Paul Nurse and Lee Hartwell, for their studies on the yeast cell cycle using *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (83).

The study of proteins involved in neurodegenerative disease have also been aided by their expression in yeast. For example, α-synuclein, a protein associated with Parkinson's disease aggregated and caused stress to yeast (84–86) possibly explaining some of its effects in neuronal cells. In addition, the yeast model has now been used to screen for compounds that ablate such activities, possibly providing new therapies for Parkinson's disease (31). Likewise, similar opportunities have emerged for the analysis of Huntington's disease (87,88).

Many other human diseases can be studied in yeast, especially where human disease genes complement those of yeast. In the case of cystic fibrosis

(CF), the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) did not directly complement the yeast *ste6* mutant. However, complementation occurred with a chimeric protein and the important CFTR Phe508 deletion caused loss of function in yeast (89), indicating the relevance of the yeast model.

7.3. Drug Research. Many approaches in yeast can lead to identification of drug targets and mechanisms. Probably the simplest example is the demonstration of a classic competitive inhibitor such as sulfanilamide (and other sulfadrugs) that compete with *p*-amino benzoate (pABA) in the folate synthesis pathway. Sulfanilamide effectively inhibits yeast growth, but the addition of pABA to sulfanilamide-treated cells (or the overexpression of pABA synthase) abrogates the growth inhibition (90).

Genomic approaches in yeast will have a major impact on drug studies for the future. One can explore drug mechanisms and targets in quite an elegant approach using genome fitness profiling as first demonstrated by Giaever and colleagues (91) and more recently by Lum and colleagues (92). The more recent example involved the screening of a "DNA bar coded" library of yeast heterozygotes with single gene deletions. Following growth of the libraries of yeast strains with separate treatments of 78 chemical entities, the bar codes of surviving cells were surveyed by gene arrays to identify the less fit strains that had become depleted during the treatments. This analysis validated known targets and in some cases identified targets of these compounds.

Gene expression profiling has already been discussed in this context.

8. Future Considerations

The major advances in recombinant DNA technology offer many new exciting possibilities for using yeast. Yeast is usually the forerunner in utilizing the new technologies and some of the advances are clearly worthwhile and life saving. However, in many cases the technology has got ahead of legislation and public acceptance so the public have uncertainties about the benefits of some genetically modified yeast in foods, for example. A possible way around this issue is to have labeling of products, but it is clear that more education is required so that opportunities are not lost and that benefits can be made.

It is likely that the public will be more likely to accept changes if they offer clear benefits, particularly in regard to health. Thus, yeast engineered to have higher folate levels might be considered advantageous. Also, although not discussed above, the ability of yeast, both live and dead, to encapsulate a wide range of compounds also offers a large range of uses. This ability has been highly exploited in the textile dye industry, however, uses can extend to insect repellents, dyes, vitamins, antimicrobials, phase change materials and in specific medical applications, antibiotics, hormones and other drugs.

Bioprospecting, the search of biota for novel valuable biochemicals, is another area of exploitation for the future. Such prospecting has commenced already for many organisms but with around 800 species of yeast and thousands of metabolites in each, it is likely that there is a wealth of products to be found in yeast. Such products may have great potential for medicine and industry and of

course with our considerable knowledge of yeast there are great opportunities to engage in the high levels of production of such compounds.

The short-comings levied on yeast are often the failure to perform a task of a native system. For example, there are differences in glycosylation between yeast and mammalian cells. Mammalian glycosylation is complex, while yeast adds carbohydrate of the high mannose type. This has particular ramifications for the production of proteins whose biological activity or immunogenicity is dependent on certain types of glycosylation. Of course, there are possible ways around these problems. Yeast deletion mutants are plentiful and complementation with human genes has been achieved in many cases. Thus production of human proteins in yeast with glycosylation resembling the native human protein is becoming achievable. The developments by GlycoFi are good examples of what is what can be achieved.

Overall, the future of yeast is brighter than ever. In particular, it would seem that its role as a model eukaryote is well understood and its applications will increase. With pathway engineering it is likely that the use of yeast as a tool for protein production will expand enormously.

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