The Eukaryote Alternative: advantages of using yeasts in place of bacteria in microbial biosensor development.

Richard M Walmsley[1] and Patrick Keenan[2]

Dept Biomolecular Sciences, UMIST, Manchester M60 1QD, UK

Tel: 00 44 161 200 4174/6

Fax: 00 44 161 236 0409

Email: [1] r.walmsley@umist.ac.uk

[2] p.keenan@stud.umist.ac.uk

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1. ABSTRACT

The relationship between Man and yeast has been a successful and enduring one. The characteristics of yeast have made it an ideal tool in scientific research and as such, it has been used extensively. In this review some of the advantages, methods and applications of yeasts in the biosensor field are outlined. In doing so, we propose a eukaryotic alternative to the current battery of bacteria-based microbial biosensors.

Key words: Biosensor, Genotoxicity, Permeability, Sensitivity, Toxicity, Yeast

2. INTRODUCTION

The term 'Yeast' usually refers to the budding yeast *Saccharomyces cerevisiae* (Figure 1). This reflects both its widespread use in foods and beverages, and its pre-eminence as a model organism, though the term simply distinguishes the single cell growth mode from the filamentous/hyphal growth mode in the Fungi.

The biosensor world has largely grown up with bacterial cells but this review reveals a healthy history of yeast exploitation. In order to limit the scope of the review, only whole cell biosensors are discussed: constituent parts of an organism become raw materials, and their relationship with the whole is not generally relevant. Whole cell biosensors are frequently, though not exclusively used as multianalyte sensors and these in turn are largely associated with environmental monitoring. Single analyte biosensors are more often developed as convenient alternatives to standard analytical chemical/biochemical techniques.

There are essentially four reasons to use yeasts. First, the 'microbial advantages' which are shared by bacteria and yeasts alike: with speed of growth, easy manipulation and growth on a variety of different carbon sources (examples including methane, amino acids, and complex sugars) at the forefront.

Second, the 'eukaryotic advantage', which is particularly significant when using a biosensor to reveal potential hazards to other eukaryotes such as humans. In this respect it is perhaps relevant to quote the Organisation for Economic Cooperation and Development in its guidelines to the well-known Ames reverse mutation test.

"The bacterial reverse mutation test utilises prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals"

Third, the 'yeast advantage'. Amongst micro-organisms, yeasts are particularly robust with a wide physicochemical tolerance and tough cell walls. These qualities are desirable in whole cell biosensors exposed to the real world. Parry [1] recently noted the physical robustness of these organisms, in comparison to bacteria, with pH tolerance between 3 and 9, survival from freezing to over 40°C, and wide tolerance of osmolarity/ionic strength.

Finally, the *'Saccharomyces'* advantage. Here, when focussing on a specific yeast, we find perhaps the best understood and most-readily manipulated of organisms. It is therefore a model for the purposes of providing knowledge of direct use in

understanding higher eukaryotes, particularly at the level of the analysis of gene function.

The wealth of yeast knowledge in the public domain makes for tailor-made organisms, which can range from those with a subtly altered native gene regulation to those expressing one or more genes from different organisms. For the biosensor scientist desirable modifications include alteration of permeability, increased specificity in analyte recognition or changes in catalytic activities, and examples of these will be discussed.

This review reflects the authors' bias towards *Saccharomyces cerevisiae* but the other yeasts are not ignored: this rather diverse collection of fungi has members with a variety of properties of use to many different biosensor scientists. It is not intended to be an exhaustive review (apologies are offered to authors whose work has not been included) and certainly does not set out to systematically compare performance parameters between different biosensors. It is intended to introduce the use of yeasts to the wider biosensor community in a representative review.

3. A BRIEF HISTORY OF YEASTS AND THEIR LIVES WITH HUMANS

This section has been drawn from several different yeast histories [2-5] and gives context to the present work. The first “interaction” between yeast and humans probably occurred in prehistoric times. The discovery of yeast, or to be more accurate, of the effects of yeast, came about by purely accidental means. Yeast, found on grapes

and grains probably fermented a neglected batch of grape juice or gruel to make the first wine and beer. It is also probable that some of this wine and beer made its way by accident into bread dough and caused the bread to rise. The French word for yeast, "levure" comes from the Latin "levere" meaning "to raise" (the English word yeast derives from the Greek "ζέω", meaning "I boil"). Throughout history, the fermentation process, driven by yeasts, has played important roles within developing civilisations. Between 6000 and 2000 BC brewing and baking was being developed in Sumeria, Babylonia and Egypt. This craft subsequently passed from the Greeks to the Romans and then to the Saxons. At the height of the Roman Empire, Rome itself was producing 500,000 loaves of bread a day. In mediaeval times, the church was the mainstay of the brewing industry, producing a potable liquid with pleasant side effects. Monasteries developed beers of different flavours, all due to the ingredients of the beer and the use of yeast strains that were occasionally unique to the monastery. The influence of the church on modern day brewing is still seen in Belgium where the brewing industry still boasts brands bearing names such as 'Trapist' after the holy order of monks who were among the early commercial brewers. Some of these strains and recipes are still in use today.

The variety of flavours within beer is testimony in part to the high degree of biodiversity. By 1998, of the 669,000 species within a probable 62,000 genera of ascomycetes [6], only 700 species had been described [7]. Hawksworth and Mouchacca [6] also calculated that it would take several hundred years for mycologists to complete the task of describing all species from a multitude of habitats.

The word ‘Yeast’ originally referred to the sediment formed at the bottom of a brew that was known to produce alcohol when a sugary liquid was added. However, little was actually known about the constituents of this residue until 1680 when van Leeuwenhoek used his newly invented microscope to study the sediment of wines and beer. He discovered that much of it was made up of numerous small spherical or oval bodies. It was only in 1835 that any association with yeast was made when Cagnaird-Latour found that these bodies were in fact individual cells that reproduced through budding. Two years later, in 1837, Meyen named a yeast that he observed in malt, *Saccharomyces cerevisiae.* In 1839, Schwann and Kützing found that sugar acted as a food source for yeast. Louis Pasteur's studies into the fermentation of barley malt sugars into alcohol, carbon dioxide and flavour compounds, led to a greater understanding of the fermentation process culminating in his “Etudes sur la bière”, published in 1876. Gradually, studies on yeast were coming more influential in the wider scientific field with the term “enzyme”, taken from the Greek “in yeast”, first used by Kuhne in 1877. The work carried out by Buchner and Buchner on the production of alcohol and CO_2 from sugar by cell-free extracts has since been proclaimed as the birth of biochemistry (reviewed in [7]). The first half of the following century saw studies by Neuberg into the production of glycerol by “steered” fermentation (1915) and Guillermond’s review (1920) of yeast physiology, sexuality and phylogeny.

The first genetic studies were carried out in the 1930’s and 1940’s by Winge, “the founder of yeast genetics” and co-workers, and Lindegren and colleagues (reviewed in [4]). The contribution that these two groups made to yeast genetics is reinforced by Kaiser *et al.* [8] who acknowledges their work as “responsible for uncovering the

general principles and much of the basic methodology of yeast genetics". During a period when *Drosophila*, corn, and *Neurospora* were the principle organisms in use, the success of yeast as an experimental organism was "dazzling" [3]. The advantageous characteristics of yeast [1,8] have been influential in furthering research in a wide variety of fields. Indeed, *S. cerevisiae* has been hailed as the preferred eukaryotic microorganism for genetic studies [3,8,9].

The modern age for yeast undoubtedly arrived with the development of methods for the transoformation of *Saccharomyces cerevisiae* with plasmid DNA [10,11]. During the 1980's and 1990's, a number of significant milestones were reached. Hepatitis B vaccine was the first commercial vaccine produced from recombinant yeast and in the early 1990s the UK government approved the use of genetically engineered yeast. Perhaps one of the greatest achievements of modern yeast geneticists was the sequencing of the yeast genome [12]. This huge feat was only made possible by the extensive genetic map started in the 1950's by Robert Mortimer and the physical map from Maynard Olsen. Completed in the spring of 1996, as the fruition of a major international collaboration, more than twelve million base pairs representing over six thousand genes were identified by computer analysis of the nucleotide sequence of the sixteen yeast chromosomes. At that point in time, it was the largest genome ever sequenced, and paved the way to the successful first draft of the human genome, announced on June 26, 2000.

The sequence revealed many new genes and, though comparisons with genes cloned and sequenced by more targeted approaches allowed preliminary suggestions for function, about 35% or 2000 genes remained with function. In response to this, the

EUROFAN (European Functional Analysis Network: 13) project was established to search for clues about the functions of these genes. Using a battery of techniques, developed for quantitative analysis of various metabolic, genetic and physiological markers, 1000 genes of unknown function were deleted, and the new mutants analysed. This study is due to reach completion by the end of 2000.

4. CHANGING YEAST GENES TO CHANGE YEAST PROPERTIES

a. Genetic engineering

The public does not always greet the use of genetically modified organisms with enthusiasm. With foods, this often reflects genuine concerns about potential health risks, though there are other concerns relating to the spread of, for example, antibiotic resistance within pathogenic organisms. The bacteria such as *Salmonella sp.* and *Escherichia coli* used in some biosensors are indeed human pathogens, so some caution has to be exercised in the use of selectable markers in modification strategies. The yeasts and yeast extract that enter the human diet in breads, beverages and food additives are not pathogenic, and only cause infections in immune-compromised individuals. As a consequence of this, the risk associated with their use in biosensors is correspondingly low.

b. Lowering the permeability barrier

The cell wall and its membrane provide physical support and a selective barrier to the entry of solutes. When using whole yeasts as biosensors it is sometimes desirable to lower this barrier. Several authors have described mutations that affect the cell wall

and increase the cells' sensitivity to toxic chemicals, including genotoxic chemicals. These genes often have the prefix MDR (Multi Drug Resistance) or PDR (Pleiotropic Drug Resistance) reflecting the acquired cross resistance of cells to a variety of unrelated cytotoxic drugs. MDR can arise through changes in a number of quite distinct mechanisms. Any one of these can be exploited to alter an *S. cerevisiae* strain.

The Venkov laboratory searched for mutations which enhanced cell permeability and increased the sensitivity of a yeast test to mutagens [14,15]. They isolated a mutant of the strain D7 (developed as a genotoxicity assessment strain: [16]) which they called D7ts1. In one study, 5 out of 14 water samples taken from the environment induced recombinogenic events in D7ts1, whereas none induced effects in wild type

The yeast (*S. cerevisiae*) cell membrane contains ergosterol instead of the cholesterol found in mammalian cells, and eleven genes specifically required for ergosterol synthesis have been identified [17]. Mutations in these genes can have severe/lethal phenotypic consequences, however cells lacking *ERG6* exhibit normal vegetative growth. They do however exhibit pleiotropic cell wall deficiency phenotypes including mating deficiency (a useful containment property) and MDR [18].

The membrane and wall contain many active proteins involved in recognition, uptake and efflux mechanisms. The ATP-dependent efflux pumps, belong to a superfamily of ATP binding cassette (ABC) transporters and mutations in their respective genes can lead to MDR. PDR5 encodes an ABC, which is under complex regulation and mutations in the gene lead to increased permeability and drug sensitivity [19,20].

The proteins of the cell wall are highly glycosylated. Interference with this process by altered regulation of the gene encoding GDP-mannose pyrophosphorylase (SRB1), an enzyme which is required for one of the earliest steps in this process, leads to MDR as well as osmotic shock sensitivity in both *Saccharomyces cerevisiae* and *Candida albicans* [21].

c. Provision of novel recognition capabilities

The ability to add and/or alter cell surface receptors and couple them to reporter systems has provided avenues for the development of both specifically engineered yeast strains, and the production of libraries of strains with altered receptors.

There has been increasing concern about the increasing exposure of humans and wildlife to pesticides and chemicals which mimic hormonal compounds. This has stimulated activity in the development of yeast tests. Several labs have reported the development of yeast cells which respond to oestrogen-like compounds (for example [22,23]) and Beresford and co-workers [24] recently reviewed the effectiveness of such tests. There are fewer reports of specific detection of pesticides by yeasts, though some preliminary data has been published [25].

John Findlay [26] at the University of Leeds has recently reported the generation of a library of yeast strains with mutants in the natural mating hormone receptor. The receptor is linked to a reporter system. He has already demonstrated that the library contains receptors even to very small molecules such as carbon monoxide. This library will doubtless be an endless source of both single and multianalyte detectors.

d. Metabolic activation

Microbes are generally deficient in the process of metabolic activation carried out in the mammalian liver. This is particularly important when searching for genotoxic activity, so rodent liver homogenates (S9 extract) are used to pre-treat test chemicals. The cytochrome P450 enzymes are responsible for many of the significant modifications of this type. Sengstag and co-workers have co-expressed human P4501A2 with a recycling oxidoreductase in yeast and successfully demonstrated *in vivo* metabolic activation, not detectable in unmodified strains. For example heterocyclic aromatic amines efficiently induced mitotic recombination [27]. Similar modifications have also been developed with bacterial genotoxicity tests [28].

5. WHAT BIOSENSORS HAVE USED YEAST?

Yeasts from a variety of different genera and an even wider variety of contexts have been used in whole cell biosensors. These are summarised in Table 1, and described below.

a. Sugar assay

Racek [29] developed a potentiometric biosensor utilising *Hansenula anomala* immobilised at an electrode surface using Calcium alginate gel. The biosensor had a useful lifetime of 2 months and was successfully used to measure glucose in urine. This is perhaps an example where enzyme electrodes (for example Medisense Inc. Waltham, MA) have in commercial terms, surpassed yeasts and other whole cell organisms. *Hansenula anomala* has also been incorporated into a carbon paste

electrode [30] which was effective in estimating L-lactate concentration. Corton and Locascio [31] reported the use of *Saccharomyces cerevisiae* as part of a carbon dioxide electrode that was used to determine the level of sucrose in dairy products.

b. Toxicity assessment

Toxicity within environmental samples can be associated with the direct poisoning of organisms or indirect effects such as oxygen starvation: many aquatic organisms are aerobic, and the digestion of biodegradeable materials can lead to acute oxygen depletion.

There are several indicators of direct toxicity to microbes, and the earliest yeast biosensors exploited changes in respiration. In 1991, Goldblum and co-workers [32] reported the use of *Saccharomyces cerevisiae* as part of a dissolved oxygen electrode that was used as an indicator of toxicity. They also reported that the choice of configuration was critical in its design, with a biofilm being most effective in their assay. This idea was developed further by Campanella and co-workers [33], who also used *Saccharomyces cerevisiae* on an oxygen electrode, but used a nutrient agar to immobilise the cells. They further developed the system measuring carbon dioxide evolution, and pH in glass and solid state electrodes [34,35]. Palmquist and co-workers [36] have also described a potentiometric method to measure metabolism in immobilised *S. cerevisia*e cells, which responded appropriately to metabolic inhibition.

The most recent direct toxicity monitor uses a rather different approach. Hollis and co-workers [37] reported the generation of bioluminescent *Saccharomyces cerevisiae*

cells, expressing firefly luciferase. Their system was conceived to overcome the disadvantages experienced with bacterial luminescence toxicity reporters. They noted in particular the stability of yeast in extremes of pH, solvent exposure, and osmotic shock. It is worth briefly describing the bacterial 'competition' in this example. The bacterium *Vibrio fischeri* has been commercially exploited in the 'Microtox™' system. This bacterium is naturally bioluminescent, and toxins reduce its ability to produce light. However, it is a marine bacterium with a narrow temperature tolerance. This means that samples have to be adjusted to saline conditions, and the assay must be performed in controlled temperature conditions: both of which affect cost. Other laboratories have addressed these problem by genetically engineering bioluminescence into less sensitive bacteria such as *Escherichia coli* [38] and *Salmonella sp.* [39-41].

Li and Chu [42,43] have described a sensor consisting of immobilized *Hansenula anomala* and an oxygen electrode for the estimation of biochemical oxygen demand (BOD). In their flow-through system, a linear relationship was observed between the relative current decrease and the BOD of brewery and glutamate-plant wastewater. Later, Preininger and co-workers [44] and Yang and co-workers [45] reported the exploitation of the opportunistic pathogen *Trichosporum cutaneum* in BOD assessment. The former describing polyvinyl alcohol (PVA) immobilised cells in the first fibre optic device in BOD measurement and the latter using a UV crosslinking resin to immobilise the cells on an oxygen electrode. Others have also used this organism [46]. Neudorfer and MeyerReil [47] reported the use of other immobilised yeasts on an oxygen electrode to assess BOD. The salt-tolerant, dimorphic yeast *Arxula adeninivorans* has been exploited by a number of groups, both as PVA-

immobilised [48] and polycarbamoyl sulfonate (PCS) -immobilised [49] components of oxygen electrodes. Lehmann and coworkers [50] has also used *Arxula adeninivorans* in BOD assessment and found that their results correlated better with BOD estimated with a commercial biosensor. Sasaki and co-workers have developed a biosensor to assess the suitability of water supplies for the sake brewing process. Whilst this is not strictly a toxicity sensor, we should all be concerned about the quality of potable alcoholic beverages! In their system, which measures alcohol evolution, it was possible to demonstrate that the harder waters used in sake factories were more efficient in supporting ethanol fermentation that the softer bottled waters tested [51].

c. Solvent assay

Chen and co-workers [52] used glycine and detergent to permeabilise the methylotrophic yeast *Pichia pastoris* in an amperometric biosensor which was sensitive to methanol and alcohol. Korpan and co-workers [53,54] used calcium alginate immobilised *Hansenula polymorpha* cells on pH and ion-sensitive electrodes to develop biosensors to alcohols with some formaldehyde cross-reactivity, but ultimately made better biosensors using purified enzymes from the same cells [55]. Gonchar and co-workers [56,57] have also used *Hansenula polymorpha* to assay alcohols. They immobilised mutants cells, either with high alcohol oxidase activity or catalase deficiency, permeabilised and immobilised in alginate gel. They were effective in the detection of ethanol, methanol and formaldehyde. A biosensor for middle chain alkanes has been developed using immobilised *Yarroiwa lipolytica* [58]

d. Genotoxicity assessment

Genotoxicity is an organism-specific and quantitative measure of the potential of a particular cell environment (chemical/electromagnetic) to cause damage to the cell's DNA. It is usually measured in order to make an assessment of the potential for that same environment to increase the likelihood of cancer in mammals. Implicit in this, the authors' own definition, is that genotoxicity assessments are not made in mammals themselves: though they can and do use mammalian cells (for example the comet test: [73]). There is a reasonable expectation from the public that chemicals entering their bodies, as food supplements and pharmaceuticals, or in tainted water and food, should not pose undisclosed health threats. A full rodent assessment of carcinogenicity costs well over a million dollars and takes up to 2 years, so such studies are only performed if there is some indication that the chemical under assessment will not cause cancer. This is why genotoxicity tests were first developed.

The first and most universally applied genotoxicity test is the Salmonella microsome reverse mutation test developed by Bruce Ames [59]. This test has undoubtedly saved many rodent and human lives. It is not described in detail here, save to mention that it takes up to five days to produce results and requires skilled microbiologists. Why seek alternatives? There are 2 reasons. First, developments in combinatorial chemistry are now allowing the pharmaceutical industry to screen perhaps millions of new compounds per year. This in turn is producing thousands of new lead compounds. At this level of demand the Ames test becomes impractical, with its high time and manpower requirements. Second, as noted earlier in this review, the use of bacteria has some inherent disadvantages. The time constraints have been addressed by a number of new bacterial tests. The Ames test itself is now produced in a high throughput microplate format (AmesII from Xenometrix: [60]). Several new tests

have been developed in which the bacterial response to DNA damage (for example components of the 'SOS' response pathway) has been linked to reporter systems. These include the SOS chromotest (reviewed in [61]) the Mutatox^{TM} test [62], the umuC test [63] and its modifications (for example [64]) and the Vitotox^{TM} test [40,41]. The outputs of these tests have been linked to measurement of chromagenic substrates as well as bioluminescence. The new tests are all geared to automation and high throughput.

Recently our lab has been addressing the more inherent problems of bacterial genotoxicty tests: the prokaryote disadvantage. A reporter for the transcriptional response to DNA damage has been developed in *Saccharomyces cerevisiae* [65, 66]. The DNA damage inducible promoter of the *RAD54* gene was fused to yeast codon-optimised gene for the jellyfish green fluorescent protein (GFP) such that cellular fluorescence increases upon exposure to a genotoxin. This test is in the development phase but our own preliminary results, as well as those from yeast reverse mutation tests by other laboratories, have already revealed the wider spectrum of carcinogen detection that yeast can provide [16, 67-70]. The test can also be performed in the high throughput microplate format [71] as well as other on-line formats [72]. More recently the *in vivo* metabolic activation system developed by Sengstag's laboratory [27] has been successfully introduced into the system (manuscript in preparation).

6. PERSPECTIVE

Yeast have demonstrated their use as model systems for basic research, and this research has provided a wealth of information and research tools that are ripe for

exploitation by the biosensor community. The challenge to yeast biosensor scientists is no different to that facing the rest of the biosensor community: to use this information to develop biosensors which can be practically and economically applied to the solution of the real problems posed by the public's demand for environmental, food and drug safety.

Acknowledgements

Nick Billinton is thanked for critically reading the manuscript.

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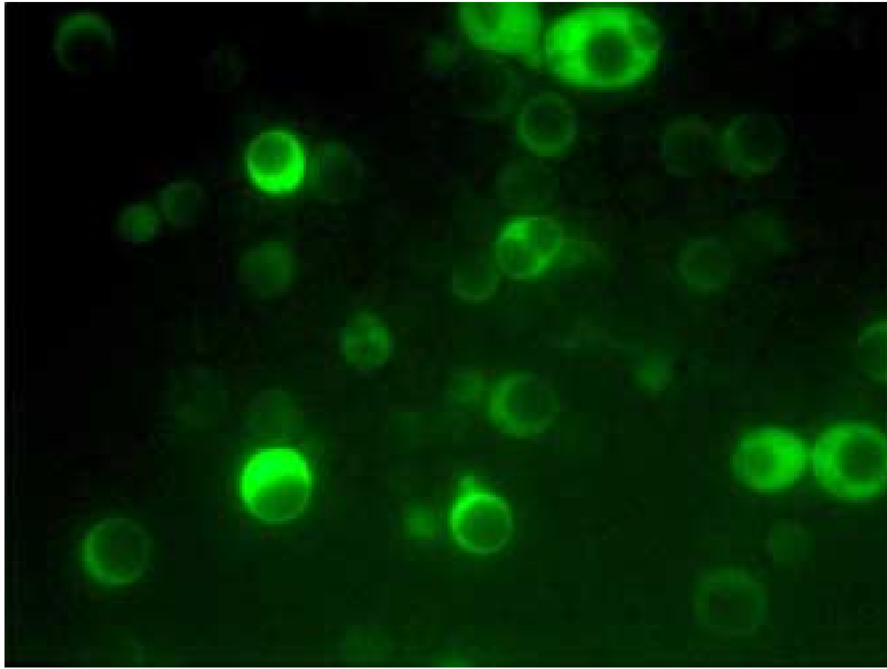

Figure 1. *Saccharomyces cerevisiae* cells expressing the Green Fluorescent Protein from *Aequorea victoria*. . Cells were viewed using a standard FITC filter set. Photo kindly supplied by Andrew Knight and Jaleel Miyan, UMIST.

Table 1. Yeasts in biosensors

Organism	Assay
Arxula adeninivorans	BOD
Hansenula polymorpha	BOD, Alcohols, formaldehyde
Pichia pastoris	alcohols
Saccharomyces cerevisiae	Toxicity, genotoxicity, BOD
Trichosporum cutaneum	BOD
Yarrowia lypolytica	Alkanes

See text for details and references.