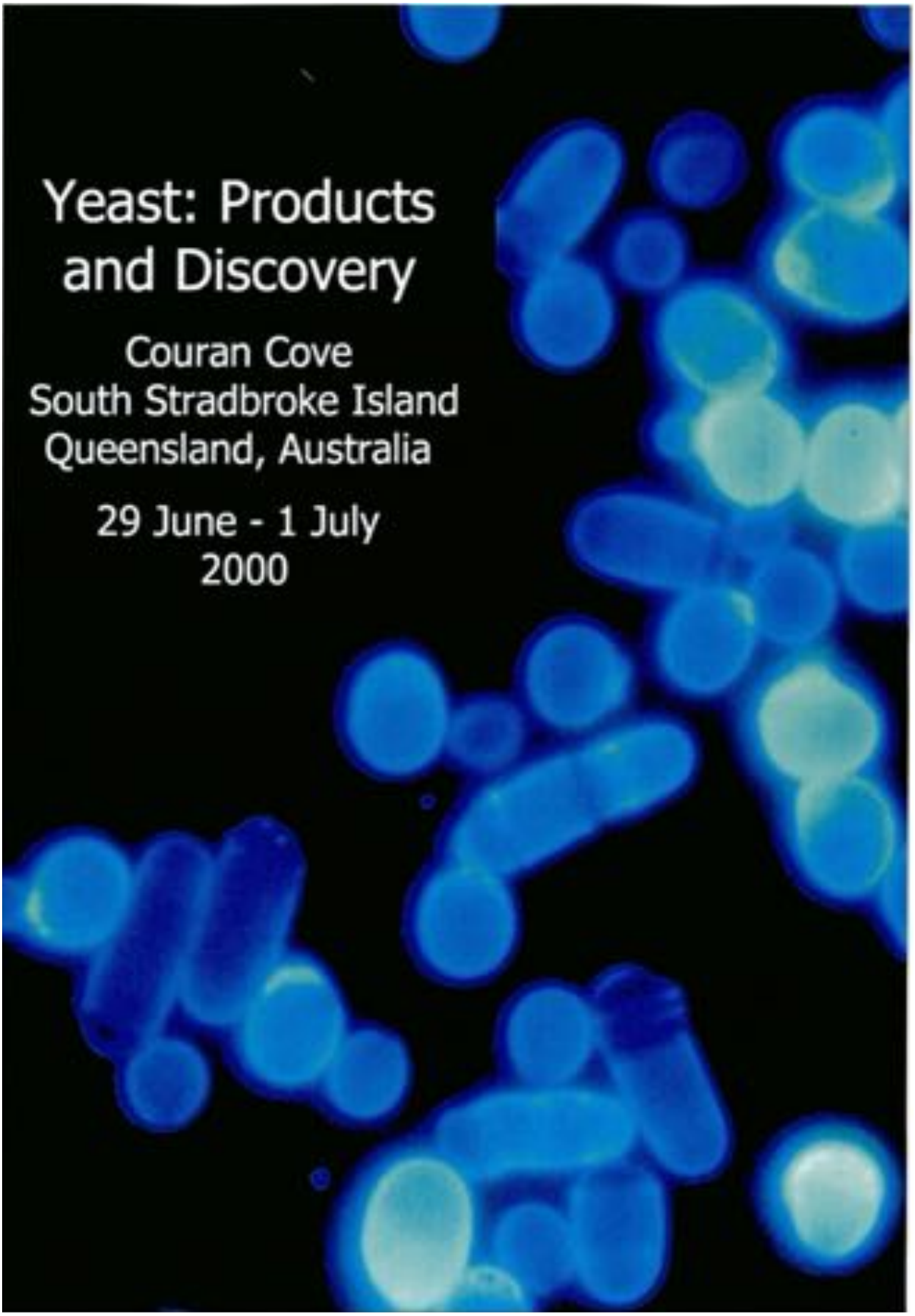
Yeast: Products
and Discovery
Couran Cove
South Stradbroke Island
Queensland, Australia
29 June - 1 July
2000

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Proceedings of the First Australian Conference on Yeast: Products and Discovery

Editor:
Dr. Paul R. Vaughan

ISBN 0 646 39435 5

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Organising Committee

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Baker's Yeast – A Stressful Business

Attfield, P. and P. Bell

Centre for Fluorimetric Applications in Biotechnology, Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

The baking of leavened dough can be traced back many thousands of years and bread remains a major staple food worldwide. In the past, the yeast produced during the manufacture of beer was used by bakers to leaven doughs, but as more breweries began to employ bottom-fermenting yeast strains there developed a need to manufacture yeast specifically for the baking industry. Nowadays, over 2 million tonnes of baker's yeast are produced each year in an industry valued at over US$2 billion.

The production of yeasts and their applications in bakeries exposes them to numerous types of stresses. These stresses include, for example, wide temperature variations, nutrient limitation and starvation, and hyperosmolarities. Fortunately, yeasts have evolved a complex set of functions that enable the cells to survive and adapt to various types of stresses. The role of stress and stress response in industrial baker's yeasts will be discussed with reference to approaches to improving strain performance.

Flow Cytometry And Fluorescence For Studying Physiology And Gene Expression In Yeast

Attfield, **P.V.,** H-Y.Choi, S.Kletsas, T.Gunasekera, A.Boyd, P.J.L.Bell and D.A.Veal

Centre for Fluorimetric Applications in Biotechnology, Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

Flow cytometry (FCM) quantitatively measures optical characteristics of thousands of cells per second as they pass within a fluid stream, single file through a focused light beam. Detection of light scattering at different angles and fluorescence at different wavelengths (colours) make possible the simultaneous measurement of multiple cell characteristics. Use of FCM in concert with fluorescent dyes and fluorogenic probes enables rapid analysis of complex events *in vivo* without the need for invasive or disruptive techniques. This potential is being exploited to monitor physiological and gene expression events in individual cells within populations of yeast exposed to stress.

Fluorescent dyes that stain cells, which have lost membrane potential (oxonol) or lost membrane permeability (propidium iodide), are being used to monitor viability of single yeast cells in real time. To permit study of gene expression in individual cells, a stress-responsive promoter (*HSP104*) has been fused to green fluorescent protein. By using dual colour FCM, it is possible to simultaneously monitor expression of the stress-responsive gene and to correlate this with stress resistance in single cells. Results of this research will be presented.

Using Flow Cytometry For Classical And Molecular Genetics Of Yeasts

Bell P, V Duncan and P Attfield

Centre for Fluorimetric Applications in Biotechnology, Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

Flow cytometry involves the passing of cells, single file in a fast moving fluid stream, through a focussed light source such as a laser beam. As cells pass through the beam information is obtained about their individual light scattering and fluorescence properties. Cells can be analysed at rates of several thousands per second. By adding fluorescent dyes or markers to cells it is possible to identify rare activities or cell types within variable populations. Furthermore, the use of fluorescence activated cell sorting (FACS) enables the isolation of an individual cell with particular light scattering or fluorescent properties.

Production of novel yeast hybrids of industrial yeasts is often limited by the inability to distinguish mated cells from parental cell types in mating reactions, because industrial yeasts carry no convenient selectable, phenotypic markers. We have circumvented the need to introduce markers to cells by using fluorescent cell tracking and flow cytometry. Thus, by staining different cells with different fluorescent dyes, we are able to employ flow cytometry and FACS to isolate hybrid cells, which become dual stained through mating.

Flow cytometry can also be use to assist in functional analysis of genes. We have constructed a promoter library containing yeast promoters fused to green fluorescent protein. By growing yeast in predetermined conditions it is possible to isolate promoters that are responsive to known physiological states and environments.

Fluorescent AFLP Analysis Of Genetic Variation In *Saccharomyces* Species And Its Application In Wine Biotechnology

Bellon[1], J., A. Heinrich[1,2], N. Shirley[3], P. Ganter[4], S. Rainieri, P. Langridge[2,3], P. Henschke[1,2], V. Jiranek[2,6], and **M. de Barros Lopes**[1,2,3]

[1]The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA, 5064, Australia. [2]Cooperative Research Center for Viticulture, Urrbrae SA 5064, Australia. [3]The University of Adelaide, Department of Plant Science, Waite Campus, SA 5064, Australia. [4]Tennessee State University, Biology Department, Nashville, TN 37209, USA. [5]University of Bologna, DIPROVAL, Villa Levi, 42100, Reggio Emilia, Italy and [6]The University of Adelaide, Department of HVO, Waite Campus, SA 5064, Australia.

Amplified fragment length polymorphism (AFLP) has been used to investigate genetic variation in *Saccharomyces* species. A number of laboratory strains, commercial yeasts and natural isolates of *Saccharomyces cerevisiae* have been studied. AFLP was shown to be effective in discriminating closely related strains. Analysis of fluorescent AFLP fingerprints demonstrate that the genome of commercial yeasts is more complex than that of the sequenced S288C strain. The polymorphic fragments detected between commercial wine strains and laboratory strains are currently being isolated and sequenced to determine if they can account for specific strain characteristics.

Fluorescent AFLP offers advantages over other molecular methods for the identification of closely related species. The method is able to separate the *Saccharomyces* sensu stricto species and estimate their genetic relatedness. Strains of these species are able to conjugate with each other, but do not produce viable spores. This conjugation permits the formation of natural hybrid yeasts which have characteristics that are distinct from the individual parental species. fAFLP has proved useful in the detection of these hybrid yeasts and in determining the relative genome contribution of each parent. This analysis will be useful in screening isolates for novel characteristics.

Profiling Ethanol Stress Response Genes In *Saccharomyces cerevisiae*.

Chandler, M., G. Stanley, P. Rogers[1], D. Emslie and P. Chambers.

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Brewing productivity and ethanol yields are compromised during fermentation due to the accumulation of endogenous ethanol. The ethanol concentration build-up acts as a potent chemical stress towards yeast cells that eventually inhibits yeast cell growth and viability. This limits the ethanol concentration in the final product and causes an increase in fermentation turnover times. The reduced cell growth rate and viability, as well as an increased growth lag period, are characteristic physiological signs of cell stress and is often accompanied at the molecular level by the induction of stress response genes. Differential Display is a PCR-based technique, based on the methods of Liang and Pardee (1992) to identify and isolate differentially expressed gene expression profiles. Our studies on stress response in *S. cerevisiae* using time-course Differential Display has identified genes that are up-regulated specifically in response to ethanol stress during the lag phase adaptation period. Yeast cells were grown in a defined medium (unstressed controls) and a defined medium with the addition of 7% ethanol (stressed). cDNA templates for Differential Display were prepared from equal numbers of both stressed and unstressed lag phase cells collected over a five hour time period. Using this method several putative ethanol-stress genes have been identified and confirmed by Northern and RT-PCR analysis. The results of this work will be presented.

Liang, P. and Pardee, A.B. (1992) *Science* **257:** 967-971.

Modulation Of Abc Transporters By The *Sit4* Protein Phosphatase

Chen, X.J.

Molecular Genetics and Evolution Group, Research School of Biological Sciences, The Australian National University, GPO Box 475, Canberra, ACT 2601, Australia

Multidrug resistance (MDR) is one of the major obstacles for successful chemotherapy of cancer as the efficacy of anti-cancer drugs is often limited by the development of a MDR system due to extrusion of the drugs through the plasma membrane. In many cases, drug efflux is mediated by an up-regulation of specific membrane transporters, such as Mdr1(P-glycoprotein) and Mrps (multidrug resistance associated proteins), that belong to the superfamily of ABC transporters. Increased activity of the transporters subsequently lowers intracellular accumulation of drugs and converts a sensitive tumour into a resistant one that no longer responds to chemotherapy. In a similar manner, many pathogenic microorganisms such as *Candida albicans* and *Plasmodium falciparum* can use the ABC transporter-mediated drug efflux mechanism to evade chemotherapy. However, little is known so far about how the activity of drug pumps is modulated at the molecular level and how cells respond to chemotherapy by increasing the activity of the drug pumps.

The single cellular eukaryote, yeast, contains several components of MDR that are equivalent to mammalian proteins. Because of genetic amenability, we have adopted the yeast system in an endeavour to unravel the principal control mechanisms of MDR in eukaryotic cells. In the budding yeast *Kluyveromyces lactis*, we found that the nuclear *SIT4* gene, encoding a PP2A-type protein phosphatase, has a broad role in controlling activity of drug transporters.

To know whether the Sit4 protein phosphatase modulates MDR by directly altering the phosphorylation state of ABC transporters, we isolated the *MDR1* gene of *K. lactis* which is responsible for efflux of oligomycin, antimycin, econazole and ketoconazole. It was found that disruption of *MDR1* abolishes the resistance of a *sit4* mutant to these drugs. It was concluded that modulation of MDR by Sit4p is mediated by ABC pumps such as Mdr1. Molecular analysis is in progress to demonstrate how Sit4p controls activity of the ABC transporters. The discovery of this fundamental regulatory mechanism for MDR could provide a target for design of drugs that can reverse MDR and improve the efficacy of chemotherapy.

Engineering Metabolic Pathways In Yeast

Dawes, I.W., C.M. Grant[2], M.D. Piper, G.G. Perrone, and A.G. Beckhouse

School of Biochemistry and Molecular Genetics & CRC for Food Industry Innovation, UNSW, Sydney NSW 2052, Australia, [2]Dept. of Biomolecular Sciences, UMIST, Manchester M60 1QD, UK

Molecular genetics techniques provide the opportunity to readily generate mutant yeast that are modified in aspects of metabolism for commercial and research purposes. Two examples will be discussed: (i) the generation of yeast strains overproducing natural antioxidants; and, (ii) the use of metabolic mutants to trace the metabolite that signals regulation of the "one-carbon" regulon in yeast, and identification of the global response of the yeast genome to this signal.

Overproduction of the antioxidant glutathione in yeast at levels that are commercially viable is not readily achieved. However, by recombinant methods deletion of the *GSH2* gene encoding the second committed enzyme in the pathway for glutathione synthesis (glutathione synthetase) has led to the overproduction of the dipeptide precursor gamma-glutamyl cysteine (GC) which is an antioxidant in its own right. GC is transported in to human cells and converted to glutathione. A *gsh2* strain has now been made by normal mutagenic techniques that has the same characteristics as the initial recombinant strain.

The availability of yeast strains with mutations in many the metabolic pathways associated with one-carbon metabolism in yeast has enabled us to show that 5,10-methylenetetrahydrofolate mediates the glycine response in yeast. This response is essentially reflects the control of transcription of a set of genes forming a "one-carbon" regulon. The extent of this regulation in cells has been determined by macroarray analysis of the entire yeast genome.

Utilisation Of Maltotriose By Commercial And Laboratory Strains Of *Saccharomyces cerevisiae*.

Day, R.E., V.J. Higgins, P. Rogers[1] and I.W. Dawes.

School of Biochemistry and Molecular Genetics, University of New South Wales, Kensington, NSW, 2052, Australia [1]Brewtech, Carlton and United Breweries, Carlton, Victoria, 3053, Australia.

Brewers yeast's performance is often limited by its inability to utilise the trisaccharide, maltotriose, compromising fermentation efficiency, resulting in extended fermentation and sub-optimal ethanol production. Commercial yeast strains grew with maltotriose as the sole carbon source. The activity of maltotriose permease and maltase were significantly higher than found in laboratory strains. The maltotriose utilisation system of these strains revealed remarkable similarities to that of maltose.

Maltotriose utilisation has been reported to be under the control of the maltose transcriptional activator. This regulation was investigated in laboratory isogenic strains with a range of maltose regulator activities. Maltotriose was not utilised in the absence of the activator. When the activator was constitutive, the maltotriose permease and maltase were highly active. This indicated that the maltose activator is involved in the regulation of maltotriose utilisation, it is yet to be established if it directly controls the system or it acts as an effector on the system.

The alpha-glucoside transporter, Agt1p, is reported to be responsible for maltotriose transport with the maltose permease having no affinity for maltotriose. We have shown this is enot the case, since a strain carrying a maltose permease gene under the control of the pyruvate decarboxylase promoter took up maltotriose at a 7-fold increased rate.

Industrial strains can ferment maltotriose, so the problem of non-utilisation is not simple, glucose repression and substrate competition cannot fully account for low fermentation efficiency. Preliminary work has shown that lower fermentation activity may be a product of the conditions present in the final stages of fermentation when maltotriose is the main carbon source.

A Functional Genomics Approach To Studying the Ethanol Stress Response In *Saccharomyces cerevisiae*

Emslie, D., G. Stanley and P. Chambers

School of Life Science and Technology, Victoria University, Werribee Campus, PO Box 14428, Melbourne City, MC8001, Australia.

Saccharomyces cerevisiae is our oldest domesticated microorganism and by far the most exploited. Despite research efforts however yeast performance remains compromised by product inhibition in one of its major applications, alcoholic fermentations. Ethanol is arguably the product with the greatest impact on yeast performance; it limits yeast growth and viability and thus limits the alcohol concentration in the final product.

We have used differential display to identify several genes upregulated during an ethanol-induced lag period in growth: YGP1, DIP5 (dicarboxylic amino acid permease) and YER024w. We have confirmed their status as ethanol stress-response genes by Northern and RT-PCR analyses. YGP1 encodes a highly glycosylated secretory protein induced by nutrient limitation (Destruelle *et al*. 1994) and during diauxic shift (DeRisi *et al*. 1997). It is maintained at a high level through stationary phase (Riou *et al*. 1997) and may be involved in cell wall synthesis (Pardo *et al*. 1999). We have used a functional genomics approach to study the role of YGP1 in the ethanol stress response by comparing the performance of the YGP1 gene deletion strain and over-expression strain with the wildtype under ethanol stress conditions during lag phase. Methods, results and possible roles of YGP1 in the yeast stress response will be discussed.

DeRisi, J. L., V. R. Iyer and P. O. Brown, 1997 Science **278:** 680-686.
Destruelle, M., H. Holzer and D. J. Klionsky, 1994 Molecular & Cellular Biology **14:** 2740-2754.
Pardo, M., L. Monteoliva, J. Pla, M. Sanchez, C. Gil *et al.*, 1999 Yeast **15:** 459-472.
Riou, C., J.-m. Nicaud, P. Barre and C. Gaillardin, 1997 Yeast **13:** 903-915.

The Biodiversity Of Yeasts In The Production Of Foods And Beverages

G. H. Fleet

Department of Food Science and Technology, The University of New South Wales, Sydney, New South Wales, Australia, 2052.

To most people, yeasts equate with *Saccharomyces cerevisiae* and the production of bread, beer, wine and distilled alcoholic beverages. These perceptions represent only a part of the broad association of yeasts with the food industry, and underestimate the diversity of yeast species involved and the potential for further commercialisation. This presentation outlines the biodiversity of yeasts connected with food production, indicating the uniqueness of their ecological, biochemical and physiological characteristics, and directions for biotechnological innovation.

While *S. cerevisiae* is responsible for most beer production, "traditional" beers are still produced in many countries and involve contributions from a range of *Candida*, *Brettanomyces*, *Saccharomyces* and *Saccharomycopsis* spp. Although *S. cerevisiae* is the principal wine yeast, most wine fermentations are the outcome of a complex yeast ecology comprising species of *Kloeckera*/*Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces*, *Metschnikowia*, *Schizosaccharomyces* and *Zygosaccharomyces*. *Torulaspora delbrueckii, Zygosaccharomyces rouxii, Kluyveromyces thermotolerans, Candida milleri, C. ohmeri, C. krusei* and *Saccharomyces exiguus* can be significant in production of breads from frozen, sweet and sour doughs. The maturation of Camembert and blue-veined cheeses involves contributions from *Yarrowia lipolytica*, *Debaryomyces hansenii* and *Kluyveromyces marxianus*. *Y. lipolytica* and *D. hansenii* also contribute to salami sausage fermentations. The multibillion dollar chocolate and coffee industries evolve from the fermentation of cocoa and coffee beans that involve a range of interesting yeast species. *Zygosaccharomyces rouxii* and *Candida versatilis* are key players in soy sauce fermentation.

Yeast extracts and autolysates, largely from S. *cerevisiae,* have extensive application as food flavourants. The composition and flavour profiles of these products could be diversified by obtaining extracts and autolysates from yeasts such as *Kluy. marxianus, D. hansenii* and *Y. lipolytica*. Some yeasts *(Sporidiobolus salmonicolor, Y. lipolytica, Candida* spp, *Cryptococcus* spp, *Kluy. marxianus)* produce lactones, esters and terpenes that are potential commercial sources of fruity aromas. Citric acid, an acidulant, can be produced by *Y. lipolytica.* The bright, orange-red, astaxanthan pigment of *Phaffia rhodozyma* has attracted commercial interest as a food colorant. *Eremothecium gossypii* and *Candida famata* are strong producers of vitamin B2, riboflavin. The glucan and mannoprotein polysaccharides of yeast cell walls represent some 30% of the cell dry weight. They exhibit a diverse range of functional properties that have potential use as thickening and gelling agents, emulsifiers, immunopotentiators, prebiotics and anti-tumour agents.

Live preparations of yeasts have applications other than use as starter cultures. Several species *(Pichia guilliermondii, P. anomala, Cryptococcus laurentii, Metschnikowia pulcherrima)* exhibit antagonistic activity towards filamentous fungi and have application in the biocontrol of fruit spoilage by these organisms. *Saccharomyces boulardii* is a potential human probiotic species, and the feeding of live yeast preparations to ruminant and non-ruminant animals can benefit their growth.

Apart from S. *cerevisiae,* few yeasts have been purposely screened for their biotechnological value in food processing. New commercial opportunities will be revealed by a systematic program of screening and strain improvement that is linked to well defined industry targets.

Identification Of Genes Contributing To A "High Nitrogen Efficiency" (Hne) Phenotype In A Modified Wine Yeast

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A key issue of fermentation is the availability of assimilable nitrogen. When nitrogen is limiting, sugar transport systems are inactivated and biomass formation is restricted. Consequently fermentation may fail to complete. The development of a highly nitrogen efficient wine yeast which catabolises more sugar with a reduced degree of nitrogen utilisation would be of great benefit to the wine making industry.

A preliminary investigation employing a laboratory yeast (209) has revealed that nitrogen efficiency can be manipulated by chemical mutagenesis. Survivors were selected in laboratory scale fermentations which were able to catabolise approximately 30% more glucose than the wild type strain in a severely nitrogen-limited situation. Subsequent evaluation in nitrogen excess media revealed that nitrogen utilisation had been reduced by between 5 and 19% compared to that of the wildtype.

To determine the impact of such reductions on other attributes of oenological importance apart from nitrogen efficiency, this study is to be continued in strains with the appropriate background. Accordingly, derivatives of wine rather than laboratory strains will be used to isolate further *hne* mutants and evaluate their effects under oenological conditions. Several strains were investigated for their suitability for this study. L-2056 (Lallemand) was selected as it sporulates efficiently with a high percentage of viable four spore tetrads. This strain does not flocculate and is able to efficiently ferment 200g/L of glucose to dryness in a chemically defined media. Since genetic studies require a heterothallic (stable mating type) yeast, a haploid strain of L-2056 has been produced by disruption of the HO (mating type switch) gene. A *ura3* marker has also been introduced to aid in phenotype tracking.

Mutagenesis is being performed utilising a transposon mutagenesis system (Ross-Macdonald et al 1997), which allows easier (PCR) identification of the genes involved in the complex phenotype of nitrogen efficiency. For analytical ease mutants will be screened in a chemically defined media initially with limiting nitrogen and excess carbon. Strains that catabolise the most sugar (as measured by refractive index) will be selected and further analysed in a media closer to an industrial fermentation, that is of excess nitrogen (750mgN/L) and limited carbon (200g/L). The genes influencing nitrogen efficiency will be identified and their precise role in nitrogen metabolism will be investigated in view of further wine yeast modification.

Dekkera/*Brettanomyces* Yeast And Mousy Off-Flavour N–Heterocycles.

PR. Grbin[1,2,3], and PA. Henschke[1].

[1]The Australian Wine Research Institute, Adelaide, SA, [2]Department of Horticulture, Viticulture and Oenology, The University of Adelaide, Adelaide, SA, [3]Current position, School of Wine and Food Sciences, Charles Sturt University, Wagga Wagga, NSW.

Mousy off-flavour is a serious spoilage phenomenon of wine and other fermented beverages. The N–heterocycles, 2–acetyltetrahydropyridine, 2–ethyltetrahydropyridine and 2–acetylpyrroline are the compounds responsible for the sensorily potent mousy off-flavor in wine. The off-flavour renders the product unpalatable and this cannot be satisfactorily reversed. Strains of *Dekkera* (and its anamorph *Brettanomyces*) yeast are associated with mousy off-flavour formation. While L-lysine has been shown to be an important precursor for the production of mousy off-flavour, the actual role of this amino acid has not been clarified. The formation of the N–heterocycles was investigated by examining the catabolism of L-lysine by *Dekkera* through several means, including dose response, sole source of nitrogen and the use of mass or stable isotope labelling. The results of these studies provided confirmation that a biological transformation was required for mousy off-flavour formation by *Dekkera*, rather than a non-enzymatic chemical synthesis. Through these investigations a biosynthetic pathway in *Dekkera* yeast is proposed.

New Generation Wine Yeast

Henschke[1,3]**,P.A.,** M. de Barros Lopes[1,2,3], J. Bellon[1], J. M. Eglinton[1,2], A. Heinrich[1,3], C. Smyl[2], A. Soden[1,2], C.M. Sutherland[1,2,3], N.A. Yap[1,2,3], P Langridge[2,3] and P. Høj[1,2]

[1]The Australian Wine Research Institute, PO Box 197, Glen Osmond, 5064, Australia
[2]University of Adelaide, Waite Campus, PMB 2, Glen Osmond, 5064, Australia
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The alcoholic fermentation of grape must by yeast is a biochemically complex process which offers considerable scope for modifying the structure, colour and flavour of wine. Wine composition is largely determined by grape (varieties of *Vitis vinifera*) berry metabolites, oak contact and the metabolic products of fungi, bacteria and yeast. Although the microbial ecology of grapes and fermenting must is now known to be highly complex and variable, fermentation with selected strains of *Saccharomyces cerevisiae* is generally preferred. The basic structural components of wine, alcohol, organic acids, residual sugar, polyols, amino acids and the phenolics, are to various degrees determined by the strain(s) of yeast. In addition, the vinous character or 'fermentation bouquet' of wine, which is composed mainly of esters, higher alcohols, carbonyls and volatile fatty acids, derives from yeast sugar and amino acid metabolism. Sensorily significant concentrations of sulfur and nitrogen containing compounds may also be formed by yeast. The varietal character of wine is, however, largely determined by a complex array of volatile grape constituents, such as monoterpenes, norisoprenoids, methoxypyrazines and phenolic compounds. A proportion of many of these compounds are present as glycosides, and these are to some extent hydrolysed during fermentation.

While yeast metabolism is subject to regulation by the nutrient and physico-chemical composition of grape must, being under genetic control, it is amenable to manipulation. Unfortunately, because many of the core properties of yeast, such as ethanol tolerance, fermentation activity and temperature characteristics, are biochemically and genetically complex, one approach is to 'reprogram' the more accessible properties of well established commercial production strains by recombinant DNA techniques. For example, we are constructing yeast with high glycerol (2-3x), and reduced ethanol and acetic acid formation, and reduced ability to form hydrogen sulfide. Experience with these projects highlights the importance of understanding the interaction and regulation of different metabolic pathways. A second approach is to select strains for desirable flavour attributes and develop technologies for their commercial use. Because these strains, for example *Candida krusei, C. stellata* and *S. bayanus*, often lack important industrial attributes, such as reliable completion of fermentation, co-fermentation technologies are being developed. The third approach under consideration is strain hybridisation where we are attempting to produce stable hybrids combining the desirable flavour attributes of the fermentatively impaired strains with the fermentative properties of *S. cerevisiae* wine strains.

Use Of Recombinant DNA Techniques To Produce New And Useful Non-Genetically Modified Yeast

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Recombinant DNA technology promised a very bright future for producing genetically modified organisms (GMO's) that were not only improvements on those that were currently used but to develop organisms that could create new industries. To a certain extent this has come to fruition with GMO's used to produce enzymes and resistant crops. In spite of these advances GMO's have a very poor perception by the media and the general public especially with its use in the food sector. Therefore the use of GMO's is not currently high on the agenda of the food industries.

Two groups of baker's strains of *Saccharomyces cerevisiae* were distinguished by the mode of regulation of maltose utilisation. The use of recombinant DNA technology determined that the *MALx3* allele present in a yeast strain and its effect on non-induced maltase and maltose permease levels was responsible for this phenotype and low second hour gas production in unsugared bread dough (maltose lag). Genetic manipulation of this target showed that the maltose lagging phenotype could be overcome without any effects on other desirable industrial traits.

The increased knowledge gained from using recombinant techniques enabled us to effectively develop selection/screening processes to isolate non-GMO mutants that displayed strong leavening abilities in unsugared and high sugar bread dough.

Total Respiration To Quicken Brewing Yeast Propagation

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Aerobic respiration of a brewing yeast strain was assessed for grow th acceleration in comparison with the propagation of the baker's yeast. Results indicate that the brewing yeast strain is able to grow on ethanol as the sole source of carbon. The yeast exhibited a growth rate approximately 2 times slower than that of gl ucose as the sole carbon source. There is also a lag phase of approximately 6 hours when the yeast is transferred from glucose to ethanol as the carbon source. Cells previously grown in ethanol had a similar growth rate but no lag phase. The lag phase can b e largely eliminated under a combination of conditions. This includes minimisation of temperature shock to the yeast cells during transfer and washing process, reducing the initial ethanol concentration to a lower level (0.5% or lower), and providing the yeast cells with an adequate amount of vitamins and amino acids. Selected nutrient supplementation neither improved the growth rate nor shortened the lag phase. The supplementation includes zinc ions as a cofactor for the alcohol dehydrogenase, L-leucine, alpha-ketoglutarate and L-malate as sources of oxoacids for the TCA cycle, and acetate as an intermediate for the ethanol catabolism.

Growth rate of the brewing yeast is similar to that of a baker's strain in using ethanol as the sole carbon source when the cells were switched from using glucose to ethanol. Effect of a higher temperature on growth rate acceleration has been investigated . Possibility and practicality of total aerobic respiration in brewing

Fluorescence Techniques For Monitoring Membrane Fluidity Modulation Of Baker's And Brewer's Yeasts

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During growth and fermentation yeasts encounter numerous changes in cellular environment, eg. variation of temperature, oxygen levels or nutrient and metabolite concentration. Yeasts may rapidly respond to such changes, provided they are of suitable viability and vitality. The primary site of response is the plasma membrane. We developed fluorimetric techniques to demonstrate that rapid membrane fluidity modulation is a component of adaptive responses of baker's and brewer's yeasts.

Analysing polarisation of the commonly used membrane probe DPH (1,6-diphenyl-1,3,5-hexatriene) we found rapid fluidity changes related to environmental stresses and cell physiology, including responses to heat, ethanol and glucose. However using this technique, cell density dependent scattering of polarised light may be problematic. Therefore we also measured Generalised Polarisation (GP) of the probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene). Laurdan and DPH based results showed similar trends, although the Laurdan technique was much less sensitive to scatter, reducing the need to precisely control cell density (which may rise during the measurement period). To extend these studies of whole populations, we used 2-photon scanning fluorescence microscopy to measure Laurdan GP of individual cells, in relation to growth physiology, heat and ethanol stress.

Thus we have developed techniques for monitoring fluidity modulation in live, intact yeasts during environmental adaptation and physiological changes. These techniques are being applied to monitoring yeasts during lab-scale pilot processing and fermentation.

Yeast Management With A Brewery And Its Impact On The Physiological Status Of The Yeast

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The management of yeast through out a brewery entails the handling of yeast through various process stages whilst ensuring that there is a continuous presence of sufficient yeast for fermentation, which is both viable and active. The various stages of yeast management and handling, comprise of yeast propagation, yeast fermentation, yeast storage, yeast transfer and waste. During these various process stages of yeast handling, the physiological status of a brewing yeast cell can be significantly influenced by a variety of environment conditions, which can have an impact on yeast health and performance. The overall health and activity of the yeast will have a significant impact on the quality, efficiency and consistency of the fermentation process. The quality and flavour characteristics of the final beer product in terms of flavour (aroma and taste) will also be directly influenced by the quality of the fermentation process.

This paper/presentation investigates the physical and biochemical changes that occur within a yeast cell during several of these yeast handling stages. From this information, an insight can be obtained to identify areas of the yeast cell which have been subjected to stress conditions or deprived of essential nutrient.

The study examines the yeast cell and changes that occur within the cell wall and membrane when the yeast is exposed to various adverse conditions during storage and fermentation. These various stress factors on the yeast can be caused by higher temperatures, high ethanol levels, variable slurry pH and limited nutrient availability over a specified time period. The impact of these conditions on nutrient utilisation and cell secretions (ie. protease release) were also investigated.

A review of current research literature is also presented to assist in better understanding the physiological behaviour of brewing yeast under industrial conditions.

The information from these studies is used to optimise the overall environmental conditions the yeast is exposed to, thereby improve the management of yeast handling within a brewery, to ensure optimal yeast health and activity, resulting in greater consistency in fermentation performance and final product quality.

Application Of Yeast For The Screening Of Antifolates

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Antifolates are among the oldest chemically-synthesised drugs and are still in common use for infection control today. Numerous bacterial pathogens and the major eukaryotic pathogens *Pneumocystis carinii* and *Plasmodium falciparum* are treated with a combination antifolates that inhibit folate synthesis and folate utilisation. However, the widespread use of these antifolates is now being limited because of drug resistant strains with mutations in the drug targets, dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). In an effort to discover replacement drugs yeast has been employed as a surrogate host for the expression of the DHPS, DHFR and other relevant genes from pathogens such as *P. carinii* and *P. falciparum*. The procedure requires the construction of knockouts of relevant genes in yeast followed by the genetic complementation of the equivalent gene of the pathogen. Such yeast can then be conveniently tested for drug sensitivity and resistance without the extreme difficulties encountered in the culture and assays of parasites. Furthermore, yeast provides opportunities to examine numerous natural and novel drug resistant alleles. In practice progress can be hampered by problems including the A+T-richness of the *Plasmodium* genome, heterologous protein interactions, and problems still to be defined. Various strategies have been, or are being, developed to overcome these difficulties. Progress to date on the development of new screens for antifolates will be discussed.

Characterisation Of Carbohydrate Metabolism By Industrial Yeast Strains Of S*accharomyces cerevisiae*

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We have conducted a survey of industrial strains of *Saccharomyces cerevisiae* to identify novel sugar utilisation phenotypes. Through an investigation of brewing, baking, wine and distilling yeasts, we have found four phenotypes which were seen to occur individually or in combination: 1. strains failing to utilise maltose 2. those having the ability to utilise maltose in the presence of glucose and/or fructose 3. those utilising maltose largely after the depletion of monosaccharides and 4. strains able to produce soluble invertase activity. These phenotypes could, in part, be attributed to deviant expression of key transport and catabolic genes. In particular, our findings indicate that constitutive maltose utilisers displayed higher initial levels of expression of the maltose permease gene at the *MAL1* loci compared to non-constitutive maltose utilisers. A similar profile was seen at the *MAL3* loci albeit at slightly lower levels of expression. In contrast, the expression of the permease gene in non-constitutive maltose utilisers only reached a maximum upon glucose depletion. The profile of net maltase and maltose permease activities during fermentation was also examined.

Yeasts Versus Bacteria For Ethanol Production From Lignocellulose

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Lignocellulosic substrates such as wood, straw and bagasse constitute a large potential feedstock for the production of fuel ethanol by fermentation. In recent years progress to develop technologies for this conversion has been extremely rapid, and several attempts to establish commercial plants are close to fruition in the United States. Probably the greatest single advance in the field has been the development of recombinant yeast and bacteria capable of fermenting the hemicellulosic portion of the substrate to ethanol. Hemicellulose, containing xylose, arabinose, mannose and galactose in addition to glucose may comprise up to 40% of the total weight of lignocellulosic materials: its efficient conversion to ethanol is vital for the economics of the process. Among the more significant recent developments in the field has been the creation of apparently stable strains of *Saccharomyces cerevisiae* containing chromosomally-integrated genes which allow the conversion of the pentose sugars xylose and arabinose to ethanol. This paper will review developments in this field and compare the performance of hemicellulose-fermenting yeasts with those of recombinant hemicellulose-fermenting *Zymomonas mobilis* and *E.coli* strains.

Modification Of *Saccharomyces cerevisiae* For The Production Of γ-Glutamylcysteine

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In the yeast, *Saccharomyces cerevisiae*, glutathione (GSH) plays a central role in protecting cells from endogenously and exogenously generated reactive oxygen species. In its absence cells grow poorly and demonstrate an increased sensitivity to oxidative stress. Glutathione deficiency in the human leads to a variety of disease outcomes. Despite this, the administration of compounds such as N-acetylcysteine has gained acceptance as a means of elevating cellular GSH levels to help prevent tissue injury and disease. Analogously, [redacted]-glutamylcysteine ([redacted]-GC) the immediate precursor to GSH, and an antioxidant in its own right, is also transported into human cells where it is converted to GSH. [redacted]-GC may therefore be suitable for use as an antioxidant.

In yeast, increased expression of the genes encoding enzymes involved in glutathione biosynthesis provides improved resistance to oxidative stress. Unfortunately increase in gene dosage does not necessarily result in elevated GSH levels during normal growth. As a consequence the development of yeast strains for the overproduction of GSH has achieved limited success. The major stumbling block has been finding a way of circumventing the regulation of the biosynthesis, which is partly due to the feedback inhibition imposed by GSH. In contrast, [redacted]-GC does not inhibit the process. Deletion of the gene encoding glutathione synthetase (*GSH2*) using recombinant DNA techniques has generated a strain that overproduces [redacted]-GC. In order to improve the commercial potential of this type of yeast strain in the current GMO weary marketplace a similar strain was also generated using non-recombinant methods.

Genome-Wide Transcriptional Analysis And Metabolic Mutations Define An One-Carbon Regulon In *Saccharomyces cerevisae*

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One-carbon metabolism plays a fundamental role in cells for the generation of purines, vitamins and several amino acids. Each of these products relies on balanced one-carbon metabolism mediated by tetrahydrofolate (THF). In order to maintain this balance, cells use a number of control mechanisms - we have focussed on this and its influence over genome-wide expression changes.
Three principle donors to the C1 pool exist in yeast: serine, glycine and formate. Glycine is catabolised by the mitochondrial glycine decarboxylase complex (GDC) and plays an important role in supplementing the C1 units derived from serine and formate for a correct C1 balance. The genes which encode the GDC were found to be induced by the addition of glycine to the medium signalled by a disturbance in C1-loaded THF molecules. Since this signal is central to C1 metabolism, the effect reflects a more widespread control and so we sought other genes which are co-regulated to determine the extent of the C1 regulon.

The use of gene array filters (Research Genetics; nylon membranes representing almost the entire yeast genome) has defined 194 genes of diverse function which have altered expression on the addition of glycine to the medium. 11 of these are directly involved in C1 metabolism. In addition to C1 gene control in yeast, these data present a model for how correct functioning of C1 metabolism in higher eukaryotes may be controlled at the level of gene expression.

Yeasts As Probiotics And Prebiotics

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Yeasts have been used for some years as probiotics for animals, including ruminants, usually dosed as dead cells. The most common commercial strain of animal probiotic is called "Yeast-sacc". The *boulardii* strain of *Saccharomyces cereviseae* has been used as a human therapeutic agent, particularly in France and USA, to treat various forms of diarrhoea. Trials with large numbers of patients have indicated the partial effectiveness of this product. This data will be discussed. Recent work in Australia has shown that certain yeast cell wall fractions, high in beta-glucans, can confer improved resistance to salmonella and clostridial infections in a mouse model. The beta glucan fraction appears to act as a prebiotic since lactobacilli populations are increased in animals fed the beta glucan. Mechanisms of action of S.cereviseae subsp. boulardii in the gut are proposed.

Modification Of *Saccharomyces cerevisiae* Yeast To Utilise Proline As A Nitrogen Source During Oenological Fermentation.

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Assimilable nitrogen is an important nutritional requirement of (Saccharomyces cerevisiae) wine yeast, which is typically lacking in grape juice. As such, wine fermentations frequently become protracted, terminate prematurely or develop undesirable aroma profiles. Amino acids, including proline, are the main form of nitrogen in grape juice.

For yeast to fully exploit proline, its transport must be derepressed by depletion of other (preferred) amino acids and molecular oxygen must be present to allow proline catabolism. Consequently, as oxygen is depleted well before other amino ac ids in grape juice are reduced to non-repressive concentrations, proline is largely unutilised by yeast despite often being the predominant nitrogen source in such fermentations.

To take advantage of oxygen early in fermentation we have produced a strain constitutively expressing the proline permease structural gene,(PUT4), to enable proline uptake despite the presence of preferred nitrogen sources.

This constitutive(PUT4)} strain was compared with a wild type, as well as a (ure2) disruptant, lacking a global repressor of nitrogen scavenging genes, such as (PUT4) . Northern blot analysis was used to confirm appropriate expression of key genes involved in proline utilisation. The constitutive (PUT4) strain and the (ure2) disruptant both transported proline in the presence of preferr ed nitrogen sources, during the initial stages of a laboratory fermentation. Poor growth of the (ure2) disruptant, however, offsets any potential benefit of proline utilisation by this strain.

Saccharomyces boulardii for The Prevention And Treatment Of *Clostridium difficile*-Associated Diarrhoea.

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Clostridium difficile-associated diarrhoea (CDAD) is a serious hospital-acquired infection that costs the healthcare system millions of dollars annually. The pathogenesis of CDAD involves disruption of the gut microflora, usually by antibiotics, followed by infection with *C.difficile*, usually acquired from the hospital environment. Although poorly understood, the diarrhoea is thought to be due to an enterotoxin known as toxin A and a cytotoxin, toxin B. Treatment with vancomycin or metronidazole is not always successful and the relapse rate is high. *Saccharomyces boulardii* is a nonpathogenic yeast that has been used to treat CDAD and prevent relapses. It survives gastric acidity and establishes in the colon, is not inhibited by antibiotics, does not impact on normal flora and has a good safety profile. *S.boulardii* produces a protease that digests toxins A and B and this may partially explain its efficacy. Over the past 2 years we have treated 15 patients with recurrent CDAD with *S.boulardii* (500 mg twice daily for a month) as an adjunct to conventional therapy. Of these, 14 have had an uneventful recovery without further relapses. Great debate is currently raging about the taxonomy of *S.boulardii* in relation to its use as a therapeutic agent. Is *S.boulardii* really *S.boulardii*, or is it *S.cerevisiae*? We have compared our strain of *S.boulardii* with other alleged *S.boulardii* strains, as well as Baker's yeast and Brewer's yeast, using PFGE. There were significant differences in banding patterns suggesting that therapeutic effect may be strain specific.

A Genetic Strategy To Reduce Sulfite Reductase Activity In *Saccharomyces cerevisiae*

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The production of hydrogen sulfide by *Saccharomyces cerevisiae* during wine fermentation has long been a problem for wine makers as H_2S has a low odour threshold. The problem occurs when yeast attempt to make the sulfur containing amino acids, methionine and cysteine via the sulfate assimilation pathway, in a low nitrogen grape juice. In the absence of nitrogen, sulfide produced by the enzyme sulfite reductase cannot combine with nitrogenous precursors to form methionine, allowing the sulfide to diffuse from the cell as H_2S.

The dominant negative approach was investigated as a genetic strategy for reducing sulfite reductase (SRase) activity so as to limit H_2S formation whilst not compromising cell growth under oenological conditions. A reduction in SRase activity rather than elimination was necessary, as grape juice contains an inadequate amount of the essential sulfur-amino acids. SRase is a heterotetramer of two α and β subunits which bind the cofactors NADPH, FAD and FMN. To reduce SRase activity, the predicted amino acids for the NADPH and FAD binding sites, present on the α subunit and coded for by *MET10,* were substituted to prevent cofactor binding whilst still allowing the α and β subunits to bind to each other. Overexpression of the altered Met10 proteins from an episomal plasmid in a haploid *S. cerevisiae* strain containing wild-type Met10p did not, however, produce the expected phenotype. Using the yeast two-hybrid assay, it was shown that the amino acid substitutions had not prevented binding of Met10p to the β subunit. Overexpression of the altered Met10 protein in a Δ*met10* strain suggested that all of the mutants retained some activity, as measured by sulfite accumulation. Explanations for the results obtained will be discussed.

A Comprehensive Molecular Study Of The Basidiomycetes Incorporating New Antarctic Yeasts

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Sequence analysis of the D1/D2 region of the large ribosomal DNA (26S) has been used to construct comprehensive phylogenetic trees for all known Ascomycetes and Basidiomycetes (Kurtzman & Rodnet, 1998; Fell et al. 2000). The adjacent internal transcribed spacer DNA (ITS1-5S-ITS2) region has also been used to identify strains within a species as has, to a lesser extent, the highly variable intragenic spacer (IGS) region. For this project, we illustrate the 26S ribosomal DNA region of the phylogenic tree for all the known Basidiomycetes with enlargement of the clades to include the newly isolated Antarctic yeasts. In the case of the latter isolates, we also include molecular sequencing data for the ITS-5S-ITS2 region. Carbon assimilation tests, 1D-proteome analyses and morphological characteristics also supplemented the molecular analyses. The vast biodiversity of yeasts isolated from Antarctica is illustrated by the identification of 11 new species of Basidiomycetes and 18 genetic variant strains, from just 36 isolates. The latter had been previously screened by 1D-proteome analyses and temperature growth profiles as psychrophilic yeasts, with a maximum growth temperature of <25°C. With further taxonomic studies in progress, this figure could be just the tip of the iceberg.

Kurtzman C.P. & Robnett C.J. 1998 Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73: 331-371

Fell, J.W, Boekhout T, Fonseca A, Scorzetti G & Statzell-Tallman A. 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large subunit rD1/D2 domain sequence analysis Int J Syst Evol Microbiol (in press)

An Overview Of Transcription In Yeast: Understanding Gene Expression For The Production Of Heterologous Products

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Production of heterologous proteins from different yeast species is now common. However there are still very few products commercially available. One of the limiting factors is the level of foreign gene products obtained. Many factors are involved in determining the level of product obtained, including the stability of the mRNA, the stability of the product, the location of the product internally in the yeast or secreted into the media, and the mechanisms necessarily used for retrieving the product from the yeast host. However, other than these considerations, a major factor that is necessary for large scale production of any protein in yeast is the level to which transcription of the foreign protein occurs and consequently the amount of the particular mRNA that is produced.

High level expression of foreign genes in yeast can be achieved simply through selection of a gene promoter that is highly expressed under the appropriate conditions. The initial choice of the promoter predicates the strategy involved for expression and determines such considerations as the growth regime, the choice of inducing agent and the timing of expression. The promoters that have been used for production of heterologous products are generally native yeast promoters. Many of the promoters that have been used are highly regulated through mechanisms of repression and activation that are controlled by different agents found in or added to the media. Given the right conditions expression can be manipulated to give high levels. A good understanding of the basic mechanisms of transcription and expression and the different regulatory controls that may be involved should enable improved means for the exploitation of yeast as a host system for the commercial production of heterologous proteins.

This seminar will provide an overview of the general features of transcription, some examples of regulatory control and systems that have been used in our laboratories to produce heterologous products from yeast, and will examine ways in which this understanding may be exploited for high level gene expression.

Acetaldehyde Stimulation Of Environmentally Stressed Yeast Fermentations: Evidence For A Mechanism

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Earlier, we demonstrated the ability of acetaldehyde to reduce the lag phase and increase the specific growth rate of *Saccharomyces cerevisiae* and *Zymomonas mobilis* cultures exposed to ethanol and temperature shocks (Stanley *et al.*, 1997). Subsequently we have observed similar effects in yeasts exposed to a wide range of organic inhibitors, including many of those present in lignocellulosic hydrolysates (Barber *et al.*, in press). These findings may have practical application in industrial and fuel alcohol production.

While the stimulatory effects of acetaldehyde have been suggested to have a physicochemical or regulatory basis, we have proposed that acetaldehyde addition functions principally to replenish intracellular acetaldehyde lost under stress conditions, thereby allowing the maintenance of an optimal NAD^+/NADH ratio and preventing NAD^+ limitation of growth. To test this hypothesis we examined the effect on growth of other compounds which promote NAD^+ formation. Propionaldehyde addition (at molar quantities equivalent to those used in acetaldehyde stimulation) greatly reduced the lag phase of *S. cerevisiae* TWY 397 and significantly reduced that of *S. cerevisiae* X2180-1A. The added propionaldehyde was stoichiometrically converted to propanol. Similar lag-reducing effects were observed using added acetoin. The data support the view that the growth stimulating effects of acetaldehyde are metabolic and attributable to its ability to accelerate NAD^+ regeneration.

Stanley, G.A.; Hobley, T.J.; Pamment, N.B. 1997 Biotechnol.Bioeng. 53:71-78

Biologically Active Preparations Derived From Spent Brewery Yeast

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Saccharomyces cerevisiae was one of the first microorganisms to be exploited in industrial processes. It is now used extensively for the manufacture of alcoholic beverages and in the baking industry.

In addition, research dating from the 1940's indicates that that yeast cell wall polysaccharides are active in stimulating the immune system in both vertebrates and invertebrates. The yeast cell wall consists of an outer mannoprotein layer, which encloses an inner skeletal layer of glucans. This paper summarises trials conducted by Carlton and United Breweries using a patented polysaccharide preparation isolated from the cell wall of spent brewery yeast.

In mice, intraperitoneally delivered yeast polysaccharides were found to stimulate the phagocytic cell population (mainly macrophages). Similar results were noted when the polysaccharides were incorporated into commercial feed and delivered to a variety of animal species, mainly chickens, fish and prawns.

Intensive commercial farming practices can lead to stress to the animals making them more susceptible to infection as a result of immunosuppression. The yeast-derived material may be incorporated into animal feeds so as to stimulate the natural immune system and has the potential to reduce stress related stock losses.

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Acknowledgments: This work was conducted at BrewTech, the technical division of Carlton and United Brewreries. The authors wish to thank the management of Carlton and United Breweries Limited and the Foster's Brewing Group for permission to publish this article.

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