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Recombinant Cytochrome P-450 Production in Yeast

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Introduction

Cytochromes P-450 (EC 1.14.14.1) are a Super-Family of enzymes, each with its corresponding gene, which occur ubiquitously in animals, plants and micro-organisms. These enzymes contain a haem component, based on FeIII plus a protoporphyrin IX, in addition to the protein component, which is usually a glycoprotein. Not less than 200 forms of cytochrome P-450 are likely to exist, many of these being isoenzymes with a relatively high sequence homology produced by closely related species, as can be displayed in evolutionary-tree diagrams (Porter and Coon, 1991).

Cytochromes P-450 are mixed-function oxidases (oxygenases) that employ molecular oxygen. One molecule of oxygen is split by the enzyme: subsequently one atom of oxygen combines with two hydrogen ions to form water, and the other atom of oxygen is incorporated into the substrate of the enzyme. This reaction often leads to the formation of a hydroxylated molecule of substrate (King and Wiseman, 1981, 1987). Cytochromes P-450 are in fact terminal electron carriers, just preceding the action of oxygen as the terminal electron acceptor, in a chain of carrier proteins. The electron and proton donor is usually nicotinamide adenine dinucleotide phosphate (NADPH), and the intermediate acceptor is a flavoprotein which contains flavine adenine mononucleotide (FAM) or flavine adenine dinucleotide (FAD). This flavoprotein is usually referred to as cytochrome P-450 reductase in animal tissues and in fungi (such as yeasts), although it is often determined as cytochrome *c* reductase by spectrophotometric measurement (*see Figure 1*). Cytochromes P-450, however, are determined by the characteristic spectral peak at 450 nm of the FeII form in the presence of carbon monoxide. This enzyme determination can be achieved even with a suspension of whole yeast cells, by a difference spectrum against the FeII form in the

Abbreviations: ADH, alcohol dehydrogenase; FAD, flavine adenine dinucleotide; FAM, flavine adenine mononucleotide; NADPH, nicotamide adenine dinucleotide phosphate.

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absence of carbon monoxide. It is this remarkably simple enzyme assay that facilitates the study of cytochrome P-450 production by yeast cultures. The reduction of the FeIII form to the FeII form is done by use of sodium dithionite, added to both cuvettes prior to saturating the contents of the test cuvette with carbon monoxide gas, followed by spectrophotometric examination in the scan range 400–500 nm.

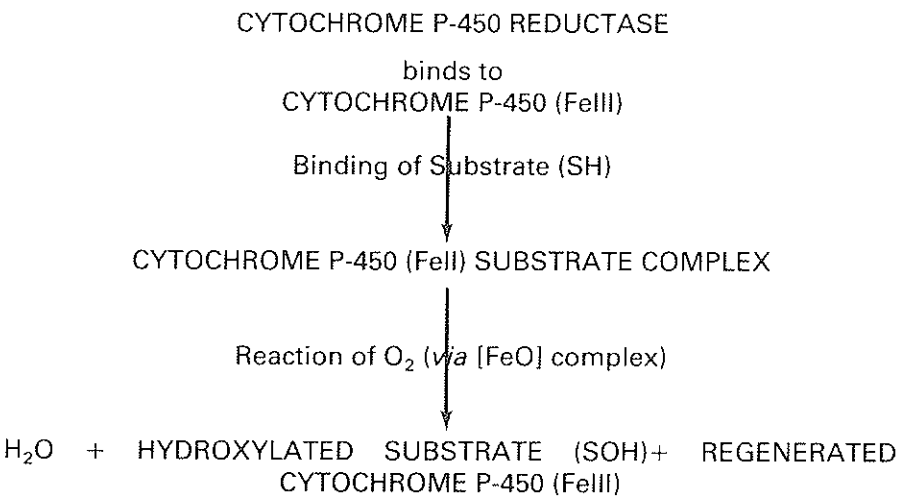

Figure 1. Outline of mechanism of substrate oxidation (hydroxylation) by cytochromes P-450. Electrons and hydrogen ions are supplied mainly by NADPH via cytochrome P-450 reductase.

Brewer's yeast accumulates a high concentration of cytochrome P-450 during logarithmic growth in glucose-rich media (King and Wiseman, 1981), especially under conditions of oxygen induction (Blatiak *et al.*, 1985). Glucose concentrations in the range 5–20% (w/v) ensure that conditions of glucose (catabolite) repression exist in the culture. Under these conditions fully functional mitochondria are not present. This is indicated by the failure of the yeast cells to complete the biosynthesis of cytochrome oxidase — one out of eight subunits of the enzyme is made in mitochondria. A similar outcome can be achieved by subjecting the yeast culture to conditions of anaerobiosis. Here ethanol produced by the yeast may be involved in cytochrome P-450 product control (Blatiak *et al.*, 1987). We have subjected brewers' yeast to thorough investigation over the course of more than 20 years to establish the enabling biochemistry for optimizing production of its cytochrome P-450. This vital knowledge can now be transferred to the exploitation of genetic engineering to produce human forms of this and other proteins for therapeutic purposes (Wiseman, 1991).

Optimization of yeast growth conditions for cytochrome P-450 production

Optimization in this context can be considered as the manipulation of the yeast growth environment to maximize the effectiveness of product forma-

tion. Before considering the optimization of any process, certain choices must be made to circumscribe the procedure.

The prime *objective of the optimization* procedure must be selected, such as maximum specific enzyme concentration or minimum enzyme cost. This may seem obvious, but some of the commonly used objectives in biological processes are mutally incompatible. High product concentrations may be associated with low production rates, for example.

Controllable process operating variables with which the growth environment can be manipulated must then be selected, such as growth temperature or glucose concentration. This may be on the basis of existing knowledge, or, more safely, on the basis of a preliminary investigation. Some variables may be controllable but have only a slight effect on the process — pH is often left uncontrolled in some commercial yeast fermentations, for example, apart from an adjustment at the start of each batch. Some variables may be known to have a considerable effect on the process, but to be very difficult to control, such as variations in complex growth-medium components. In addition, operating variables are rarely independent of each other, and their interactions need to be allowed for in the optimization procedure chosen.

The extent or complexity of the *optimization procedure* must then be chosen, which is usually determined by the time or resources available, whether the expected improvement justifies the resources used and whether, for example, a rapid rough answer is more appropriate than a more accurate result from a long-term investigation. A long-established process is likely to have been gradually optimized by trial and error over several years, and a superfical analysis may not come up with further improvements. Such experiential, as opposed to experimental, improvements will however, have dealt only with situations which have actually occurred, whereas a suitably designed optimization procedure may be able to allow for previously unencountered situations. For example, if a fault in the temperature control system means that the precise optimal operating temperature cannot be maintained, then it may be possible to prescribe a modified growth-medium formulation or air flow-rate which will be optimal at that new temperature, and partly compensate for the loss in process performance. It should also be borne in mind that the process control strategy arising from an optimization procedure may be impossible to carry out in practice, such as an instantaneous change in substrate feed in fed-batch operation.

The most appropriate optimization procedure must then be chosen. The essence of an optimization procedure is to carry out a sequence of experimental fermentations in which small but significant changes are made in the settings of the selected controllable operating variables according to a pre-determined pattern or *experimental design*. The responses to these changes in terms of the selected objective are measured, and the combination of settings of the variables tested giving the most favourable value of the objective is determined. This procedure is then repeated in a systematic pattern; the results indicate the changes in operating conditions which will move the system in the direction of the most favourable value of the response — highest yield, lowest unit cost and so on. A suggested analogy (Winkler,

1991) is of finding the most efficient means of heating a room by measuring temperatures at different positions in the room space while testing different combinations of heater output, heater location, ceiling and wall insulation and floor covering. A process being optimized can then be considered as a 'space' bounded by a set of 'dimensions', with each operating variable — temperature, pH, nutrient concentration and so on — constituting a 'dimension'. The optimization procedure is then a systematic search to find the position within that 'space' which has the most desirable process response and the value of the response.

Clearly, computer simulations offer the fastest and cheapest vehicle for process optimization, but their usefulness depends on the accuracy of the mathematical model of the process upon which they are based. Conversely, a programme of experimental fermentations is very time consuming, but will produce useful amounts of product at the same time as providing the information required for optimization. The ideal method would thus appear to be a well chosen computer model which is continually updated by experimental process data.

Factor analysis

Whichever optimization procedure is used, a set of controllable variables with which the process is manipulated must be selected. Ideally, all controllable variables known to influence the process should be tested for their effects on the selected objective, individually and in terms of their interactions with each other. In an experimental optimization procedure, this could result in an enormous amount of experimentation and data analysis, as the number of experiments required increases exponentially with the number of variables tested. Even with computer simulations, economy in the demand on heavily used computing facilities is desirable. At the same time, it is even more important that a key variable is not left out of the optimization procedure, as this could invalidate its results. It is therefore desirable to limit the variables investigated to those whose effects and interactions are known to affect the response of the process significantly, compared with the usual random variation. These may be selected from knowledge and experience of the process, where it is sufficiently well understood, but it is safer to carry out a quantitative investigation. A procedure known as *factor analysis* has proved to be useful in this respect.

The problem of selecting appropriate controllable process variables for inclusion in an optimization procedure is that the variables are not usually independent of each other. For example, a change in glucose concentration will also change the solubility of oxygen in the medium, the viscosity and the osmotic pressure of the medium, and thus dissolved-oxygen availability and homogeneity, as well as carbohydrate availability. Thus the 'optimum' setting of a variable depends on the values of the other variables affecting the process. Factor analysis gets round this problem by assuming that there are a similar number of hypothetical variables which are truly independent of each other, but which are affected by the real controllable variables. These

hypothetical independent variables are called *reference factors*, and because they are by definition independent of each other, the effect of an individual reference factor on the process is clearly identifiable. The reference factors then contribute differently to each non-independent experimental variable, according to a different formulation or *vector* for each experimental variable.

The factor analysis procedure is to carry out a set of experiments whose results can be used in determining the composition of these vectors. The reference factors are purely hypothetical, but can sometimes be considered as having realistic properties. For example, a reference factor contributing large proportions of the effects of agitator speed and configuration could be a 'homogeneity factor', while one contributing to the effects of dissolved oxygen concentration and air flow-rate could be an 'oxygen availability factor'. The reference factors are ranked in order in terms of the statistical significance of their effects on the chosen objective of the process. The experimental variables tested can then be placed in order of their effects on the process from the ranking of the reference factors and the formulations of each variable in terms of the reference factors. Experimental variables which are formulated mostly from reference factors which are highly ranked and have highly significant effects on the process objective are clearly important and therefore should be included in an optimization procedure; those made up of low-ranking reference factors may be left out of the procedure. Similarly, the number of reference factors common to two or more experimental variables indicates the extent of the interactions between them, while the ranking of the reference factors involved indicates their importance. A fairly non-mathematical outline of this procedure has been given by Winkler (1988).

In the optimization of culture conditions for production of cytochrome P-450 by *Saccharomyces cerevisiae*, factor analysis was used in selecting the variables used in the optimization procedure (Salihon, Winkler and Wiseman, 1983). The optimization was being carried out as a preliminary stage of scaling up the fermentation from shake-flasks to a 4-litre stirred-tank system: there is clearly no point in scaling up a non-optimized process. In addition, the planned increase in scale meant a corresponding increase in the cost of growth medium. To find a cheaper formulation of growth medium, one of the objectives chosen for the optimization was to maximize yield of cytochrome P-450 per unit cost of growth medium. The most expensive medium component was mycological peptone, and it was proposed to replace this partly by cheaper yeast extract. However, the yeast extract appeared to have a higher salt content than mycological peptone, and factor analysis was used to find out whether the change in salt content was significant. Six variables were tested in shake-flask experiments: temperature, initial pH, and initial concentrations of glucose, peptone, yeast extract and salt. The results were analysed to find the significance of their effects on total enzyme yield, enzyme yield per unit cost, biomass yield and specific enzyme yield. The salt concentration was shown by the factor analysis to be unexpectedly important and would probably not otherwise have been included in the subsequent optimization procedures. Overall, each variable tested had a statistically significant effect

on at least one of the objectives, although not all variables had a significant effect on all process objectives. The subsequent optimization procedure was therefore designed to include all six variables tested in the factor analysis. The review mentioned earlier (Winkler, 1988) also includes an outline of factor analysis in optimizing the utilisation of whey by *Kluyveromyces fragilis*. Discussed below is a statistical approach advocated by Taguchi which can be considered as combining factor analysis with factorial experimental design.

Single factor optimization

The simplest form of optimization is to maintain all controllable variables in a process except one at their usual settings. The process responses obtained with different settings of the remaining variable are determined, and the setting giving the most favourable response is taken as 'the' optimum setting of that variable. The process is then repeated for each of the other process variables in turn, so that a set of 'optimal' values is obtained for the process. This method has the advantage of being straightforward to plan, analyse and interpret, and can give a rapid answer where the ranges of the variables tested is narrow, which is usually the case in biological systems. However, the method is valid only if the variables are independent of each other and there is no significant random and uncontrolled variation in process conditions. As discussed in the section on factor analysis, this is very unlikely to apply to biological systems, and the values obtained will depend on the order in which the variables are tested. The procedure can be repeated several times in the hope that the results will 'home in' on a set of true optimal values, but if there are strong interactive effects, then the results are likely to be confusing and seem contradictory. In addition, the extra testing will take up resources which would be more rewardingly expended on a more sophisticated statistical design, such as factorial experimental design. Single factor optimization is useful, however, as a rapid means of finding a suitable starting point for the more systematic optimization of a new process for which there is no obvious set of standard operating conditions.

A process for the production of human proinsulin in *Saccharomyces cerevisiae* has been successfully scaled up to fed-batch operation in a 200-litre fermenter using fairly straightforward optimization principles (Tøttrup and Carlsen, 1990). Formation of human superoxide dismutase-human proinsulin fusion product was regulated by a hybrid ADH-glyceraldehyde-3-phosphate dehydrogenase promoter, which was repressed by glucose and derepressed by glucose depletion. The optimized fed-batch process was considered suitable for further scaling up to cubic metre level. The optimal temperature for secretion of non-glycosylated human prourokinase, single-chain urinary plasminogen activator, from *Saccharomyces cerevisiae* was determined in a version of fed-batch fermentation called 'perturbed batch' fermentation (Turner *et al.*, 1991). It was concluded that the conditions optimal for biomass productivity are not necessarily optimal for secretion of heterologous proteins from yeast.

Factorial experimental design

A means of assessing the relative importance of individual process variables and of their interactions on the response of a process is offered by a statistical procedure called *factorial experimental design* (not to be confused with *factor analysis*). The design consists of a series of experiments in which the effects of different combinations of alternative values of the experimental values are measured and analysed. The alternative values tested can be numerical values, such as temperature, pH, glucose concentration and so on, or non-numerical 'either/or' conditions, such as alternative strains of organism or different impeller configurations, or 'yes/no' situations, such as with and without a suspected micronutrient or an antifoam agent. If all possible combinations of values are tested, then the total number of experiments needed is equal to the number of test levels raised to the exponent of the number of variables tested. For example, if four variables were each tested at two levels — say, usual value and a higher value, or yes/no, then the total number of different combinations is $2 \times 2 \times 2 \times 2$ or $2^4 = 16$ combinations; if three variables were tested, two at two levels and one at three levels then the total number of combinations would be $2^2 \times 3^1 = 12$. In addition, the high degree of variability means that each combination needs to be tested several times, called multiple replication, to ensure that the effects of process modification are not confused with uncontrollable or random changes. This means that investigating only two alternative values of even a small number of variables can result in an enormous amount of experimentation if all possible combinations are tested. In a two-level investigation, each additional variable tested doubles the number of possible combinations, which emphasizes the value of a preliminary screening procedure for variables, such as factor analysis.

To avoid the risk that historical trends might influence the interpretation of results, the experiments in a design are performed in random order. Any historical trend, such as deterioration of stocks of growth-medium components or changes in stock cultures of organisms, then appear as contributions to experimental error rather than being associated in a misleading way with changes in a particular test variable. This does however have practical disadvantages, because if for some reason, such as equipment failure or withdrawal of resources, the experimental plan has to be abandoned before the design is completed, it can be difficult to extract useful information from an incomplete randomized design. One of the present authors was caught out by this while optimizing an industrial downstream processing plant. The experimental design was planned to take about three months to complete, and so a certain amount of embarrassment ensued when results were demanded after a fortnight! Had the requirement for such a rapid response been known at the outset, then a rough single-factor optimization plan would at least have given some sort of answer in that time. A method of allowing for historical or equipment differences is known as *confounding*. The experiments are designed in a number of sets or *blocks*, in such a way that the block-to-block differences are associated with effects which are of little

interest, such as multi-variable interactions, because the information on both them and their aliases is lost by confounding with blocks.

FRACTIONAL EXPERIMENTAL DESIGN

If every possible combination of variables is tested once, then every possible interaction between variables is tested at least once, but the main effect of each variable individually is tested many times over. For example, in a 2^6 full factorial, with all 64 possible combinations of six variables each at two different levels, the main effect of each individual variable is effectively tested 32 times, which may be considered more than ample replication. The total number of experiments can be reduced by using a *fractional design*, in which the main effects of each variable and their principal interactions are tested with a reasonable number of replications, while the effects of multiple interactions are sacrificed. Considerable care needs to be taken in the selection and analysis of fractional designs, particularly in ensuring that the number of experiments carried out gives enough information or *degrees of freedom* for the estimation of experimental error. This can cause problems even in a full, non-fractional factorial design if only one replication is carried out. Another hazard is the existence of a non-required effect, called an *alias*, whose value is taken from the same data as a required effect.

Sets of experimental designs have been published, and particular interest has been aroused recently by fractional factorial designs due to the Japanese statistician Genichi Taguchi (*see* Winkler, 1988, 1990, 1991). His designs are set out in tabular form which simplifies data analysis and is also well suited to calculation on PC spreadsheets. These can be used quite conventionally, as with any published experimental designs, with awareness of the pitfalls of fractional designs already mentioned. The tabular format is so well set out that a good estimate of the relative effects of the variables tested can be obtained just by inspection of the processed data. Particular attention has been focused on an application which, in effect combines factorial experimental design with factor analysis. As many variables as may be considered relevant are included in a design, and the variables contributing only minor effects to the response after analysis are assumed to be non-effective and their contributions are 'pooled' into the estimate of experimental error.

OPTIMIZATION OF CYTOCHROME P-450 PRODUCTION BY *Saccharomyces cerevisiae*

In the investigation of cytochrome P-450 yield from *Saccharomyces cerevisiae* (Salihon, Winkler and Wiseman, 1983) the six variables shown by factor analysis to be significant (temperature and initial pH and concentrations of glucose, mycological peptone, yeast extract and salt) were each tested at two levels in two successive 2^6 half-replicate factorials in shake-flasks. This was to optimize the growth conditions in shake-flask culture prior to scaling up to 4-litre stirred tank fermentation. The culture conditions used by the collaborative group that developed the high-yielding yeast strain (Blatiak *et al.*, 1985)

provided the basis for the combination of values of the six variables tested used as the *centre-point* or starting point for the first 2^6 half-replicate factorial design. Analysis of the results, using a technique known as the *method of the path of steepest ascent*, indicated the directions in which the values of the variables needed to be changed in order to improve the yield responses. A new centre-point combination of values was selected on which the second half-replicate 2^6 factorial design was based. This produced improvements in yield, but additional information was needed to confirm whether this represented an optimum set of conditions. It is not possible to identify a maximum in experiments with only two levels used. Fortunately, a technique called *rotatable composite design* enables information from a two-level design to be utilized in inferring the presence of an optimum by testing a limited number of additional combinations, provided the two-level design is close to optimal conditions and that the experimental error can be assumed to fulfil certain conditions. This saves carrying out a new design at three or more levels. In this case, the second centre-point proved to be reasonably close to the optimal set of conditions, and all the variables tested proved to be significant in describing the optimum. Relative to the original starting point, the optimum conditions gave an increase of 35% in the total yield of cytochrome P-450 and a 56% decrease in enzyme cost, at the expense of a 7·5% decrease in the specific enzyme concentration in the biomass. The process response was further analysed, using the mathematical technique of *canonical analysis*, which simplifies the mathematical equations describing the process response. A set of conditions was identified which reduced the loss in specific enzyme yield to 6·5% and increased the total enzyme yield to 40% compared with the original conditions, but decreased the enzyme cost reduction to 53%. Overall, the optimization procedure increased the productivity of shake-flask culture by nearly half as much again and decreased the cost of the cytochrome P-450 produced by more than half, while producing *S. cerevisiae* biomass with roughly the same specific concentration of cytochrome P-450. This was then used as the starting point for scaling up to a 4-litre stirred-tank process.

Scaling up the optimized shake-flask culture to obtain optimal stirred-vessel conditions presented three new problems. First, our collaborative research group had resolved the yeast culture into two strains, low-yielding and high-yielding in cytochrome P-450, designated *S. cerevisiae* NCYC753 and NCYC754 respectively. The higher-yielding NCYC754 was used in further work. It was presumed that the preliminary optimization procedure had worked, at least partly, by finding growth conditions favouring the higher-yielding strain. Second, stirred-vessel operation involved two additional variables, the input air flow-rate and the agitator speed. Third, a different inoculation technique had to be used.

Air flow-rate and agitator speed can be considered as involving a hypothetical 'oxygen availability' or 'oxygenation' factor as well as a 'homogeneity' factor. As stated in the *Introduction* to this chapter and elsewhere (Dorr *et al.*, 1988; Sariaslani, 1991), cytochrome P-450 is induced after 'semi-anaerobic' or 'micro-aerophilic' growth on glucose. This involves maintaining a dissolved oxygen concentration of about 0·01 g m^{-3}, which was too low to

be controllable by conventional means in a stirred-vessel growth system. Clearly, some oxygen had to be made available to the yeast, by aeration and agitation, so air flow-rate and agitator speed were included in the vessel optimization procedure. In effect, this was using the oxygen transfer coefficient as an optimization variable. A starting value was obtained by immersing a dissolved oxygen probe in sterile optimal growth medium in a shake-flask and estimating the oxygen transfer coefficient obtained in shake-flask culture. A suitable combination of values of air flow-rate and agitator speed was then calculated from one of the many empirical correlations describing oxygen dissolution in agitated air-sparged vessels (Winkler, 1990).

Inoculation of a 4-litre vessel in the same way as a shake-flask, with a wire loop, is not only mechanically awkward, but also wastes fermenter time. It was therefore decided to grow up seed cultures in shake-flasks and inoculate after a standard growth time of 48 h. The quantity of inoculum used was included as a variable in the optimization procedure.

Inclusion of three additional variables implies carrying out eight times the number of experiments. To reduce the number of variables tested, it was assumed that the growth medium composition optimized in shake-flask culture would also be close to optimal in stirred-vessel culture. Although valid on nutritional grounds, this assumption may be questioned on grounds of oxygen availability, because the growth medium solutes will affect the viscosity and density of the liquid, and thus the effects of agitation. It was argued, however, that the optimal air flow-rate and agitator speed obtained from the optimization procedure would be the values *for that medium composition* and therefore acceptable. The variables selected for optimization were then temperature, controlled pH, initial glucose concentration, agitator speed, air flow-rate and inoculum level.

The first factorial experimental design used in stirred-vessel growth was a half-replicate 2^6 factorial, using the shake-flask optimal conditions as the starting point. This did not contain the optimum, but did show that air flow-rate and agitator speed were the most significant of the variables tested and that the two inoculum levels tested were not significantly different. Air flow-rate and agitator speed were then tested separately in a 2×2 factorial design, based on values worked out from the results of the 2^6 half-replicate design using the method of the path of steepest ascent. The steepest ascent method was again applied to work out the centre-point for a 2^4 half-replicate design involving pH and temperature as well as air flow-rate and agitator speed. A further steepest-ascent search based on these results indicated the existence of a high-yielding set of conditions, which was replicated six times and used as the centre-point of a $2 \times 2 \times 2$ factorial involving temperature, pH and agitator speed as variables. This design looked as if it could contain the optimal set of conditions, so additional experimental combinations were tested to complete a rotatable composite experimental design. The theoretical optimum indicated by the results proved to be close to the centre-point of the original factorial design. Compared with the original conditions, the improvements obtained were an increase of 173% in total yield of cytochrome P-450 in the culture and an increase of 255% in specific enzyme yield per unit

weight of biomass. Part of this improvement must be ascribed to the more efficient process control obtainable in stirred-vessel fermentation than in shake-flask culture and the use of the high-yielding strain of *S. cerevisiae*.

Factorial experimental design was used to optimize the stereoselective reduction of ethyl acetoacetate to (+)-(S)-ethyl-3-hydroxybutyrate, a precursor for the antibiotic griseoviridin and for (S)-(+)-sulcatol, an insect pheromone, by *S. cerevisiae* in batch culture (Boccù *et al.*, 1990). Complete substrate conversion was achieved, with more convenient operating conditions. The review cited earlier (Winkler, 1988) includes a summary of a similar procedure applied to the utilization of whey by *Kluyveromyces fragilis*. The procedure has also been used to optimize the conditions for immobilizing *S. cerevisiae* in alginate gel, giving a 75% increase in ethanol fermentation efficiency (Jamuna *et al.*, 1992).

Variability

Biological processes in general are well known for their variability in performance, particularly in batch-to-batch variation in nominally identical procedures. This is a particular nuisance in optimization because high levels of replication in experimental work are needed to separate the results of deliberate process changes from random process fluctuations. In addition, process variability creates considerable difficulty in establishing reliable and useful mathematical models of fermentation processes for the purposes of control and optimization. The traditional technological approach to variability is to investigate the causes of variability and correct the fault. In biological systems, however, the causes of variability may be well known, and are usually attributed to variations in the composition and quality of complex raw materials and in the strain of the organism. There may be little direct action to correct such *causes* of variation, so a method of limiting or suppressing the *effects* of these is needed. This approach has proved very successful in manufacturing processes, following methods pioneered by Genichi Taguchi in Japan (an outline of the Taguchi method is given by Winkler, 1991, 1990, 1988). The present authors have applied the Taguchi method to the production of cytochrome P-450 by *Saccharomyces cerevisiae* with, at the time of writing, promising results. The investigation principle used by the present authors was to consider the *variability* of cytochrome P-450 yield as a process response, in the same way as the yield itself. The effect of different sets of operating conditions on the variability was then investigated by factorial experimental design, using one of Taguchi's designs. The principle problem is that high levels of replication are needed to assess variability in a highly variable process, necessitating large numbers of experiments.

Computer optimization

Numerical simulation appears to offer a cheap and rapid means of process optimization, but requires a reliable mathematical model of a process on which to operate. In addition to the characteristic variability of biological processes

in general, batch processes involve continually changing conditions, while continuous processes apparently at steady-state usually contain a population of cells with a range or distribution of ages and behaviour. All these effects need to be allowed for in the model used. General models for aerobic yeast growth have been developed for batch growth (Barford, 1990a) and continuous culture (Barford, 1990b), based on relative transport rates across mitochondrial and plasma membranes. The batch model was shown to be consistent with a wide range of experimental data from batch culture of *Saccharomyces cerevisiae, S. uvarum* and *S. pombe*, while the continuous model successfully simulated the complete range of metabolic responses exhibit by yeast grown in continuous culture, such as respiratory repression, saturated respiratory capacity and respiratory depression.

The use of *S. cerevisiae* in recombinant DNA technology has several advantages: the organism has a highly developed capability for genetic manipulation; it is robust and well suited to industrial production; and it is one of the few micro-organism that are *generally regarded as safe* (GRAS) by the United States Food and Drug Administration (FDA). Attention is now being given to the modelling of recombinant yeast growth and product formation. As the efficiency of expression of heterologous protein products depends on the number of copies of the recombinant vector in each cell, the distribution of a plasmid in a baker's yeast strain of *S. cerevisiae* was studied using a flow cytometer (Wittrup *et al.*, 1990). The results showed a biphasic distribution of plasmids, such that a population with an overall average copy number of 13–14 contained a significant proportion of cells with a copy number of 4–6. From the results, a mathematical model based on the probability of plasmid loss at cell division was developed which successfully predicted the biphasic distribution. Plasmid-carrying yeast cells can now be rapidly determined using an imaging sensor system (Endo *et al.*, 1991).

CLOSED-BATCH FERMENTATION

In the production of cytochrome P-450 by *S. cerevisiae* in closed-batch fermentation optimized statistically by the procedures described above, the optimal values of controllable variables were those which were maintained at constant values throughout the course of the batch, termed *set-point control*. Other process conditions, such as nutrient and product concentrations, vary continually throughout the batch as substrates are consumed or converted and products are generated. It may be supposed, therefore, that there is some pattern of change of process variables through the course of the batch which gives rise to the most desirable result, such as maximum product yield. This pattern is known as the *trajectory* or *time-profile* of each variable, and can be computed numerically from a numerical model of the process. If the process obeys the numerical model and each process variable is controlled according to its optimal profile, then the most desirable result should be obtained. This approach has been reasonably successful in optimizing production of penicillin, gluconic acid from *Pseudomonas ovalis* and erythromycin from *Streptomyces erytheus*. Outlines of the optimization procedures and the numerical

models involved have been presented (Winkler, 1991). The present authors' group has investigated time-profiling of the production of cytochrome P-450 by *S. cerevisiae*. The time-profiles of three controllable variables — temperature, pH and agitator speed — were computed by applying the Pontryagin continuous maximum principle to a simplistic model of cytochrome P-450 production, using the Logistic equation to describe yeast biomass growth and the Lüdeking equation to describe cytochrome P-450 formation (Salihon, 1984; Grob, 1991). The model predicted a doubling of cytochrome P-450 yield in time-profiled fermentations. Although yields were good compared with constant set-point controlled fermentations, this predicted improvement was not achieved. This was attributed mainly to the simplicity of the models used, the limitations of the computer control program in generating the prescribed profiles, and the inherent problem with pre-determined control strategies in biological systems — once the system deviates from the prescribed trajectory, the remainder of the trajectory is invalidated.

FED-BATCH FERMENTATION

Fed-batch fermentation is used for several important industrial biological processes, including the production of baker's yeast, penicillin, enzymes and organic acids. Some success has been obtained in computing feed time-profiles based on empirical and other mathematical relationships describing biomass and product formation (Winkler, 1991). The feeding pattern of nutrients in baker's yeast production is extremely important. Sufficient nutrients have to be added to maintain maximum yeast productivity, while excessive nutrient supply which does not match the availability of dissolved oxygen to the yeast will produce anaerobic conditions with concomitant wasteful ethanol production. The baker's yeast fermentation is consequently a firm favourite with investigators of optimization and control techniques. Fed-batch fermentation is especially useful where substrates involved have an inhibiting effect on the process, so that they can be added to the system only at a rate matching their consumption or depletion. Other classic examples include the addition of the toxic penicillin-G precursor, sodium phenylacetate, to penicillin fermentations, and the continuous feeding of oxygen to aerobic fermentations because of its low solubility in growth media.

The pattern of feeding fresh liquid growth medium to the fermentation is important because the medium-feed-rate becomes a key process variable. Addition of fresh nutrient medium increases nutrient concentration and thus increases biomass growth and product formation activities, while diluting the biomass and product already present. Various feeding patterns may be used in fed-batch fermentation, as some materials may be supplied continuously, as air usually is, while others are supplied intermittently. Some of the culture may or may not be removed from the fermentation to make room for the feed. When no culture is removed until the completion of the batch, as is usual in fed-batch baker's yeast production, the system is sometimes called a 'single fed-batch' process. This has the disadvantage that a proportion of the working volume of the fermenter vessel has to remain unused for much of the

process with a consequent loss in productivity. When volumes of culture are removed periodically from the vessel and replaced by fresh medium, as in penicillin production, the system is sometimes called a 'repeated fed-batch' process. A repeated fed-batch system where the feed additions are frequent approximates to a continuous system and may be expected to give maximum productivity of growth-associated products. A process where part of the culture is left in the vessel at the end of the batch to act as an inoculum for the next batch is called a 'fill-and-draw' system. Fill-and-draw systems are used in biological waste-water treatment and have also been used successfully in the production of monoclonal antibodies. There are dangers in using fill-and-draw operation for eukaryotic organisms, however, as the imposition of sequences of plentiful and depleted nutrients is a standard method of inducing synchronous growth.

Mathematical analysis of both single and repeated fed-batch operation indicates that the optimal productivity depends on the ratio of the starting volume to the final volume in the fermenter. The latter is, of course, determined by the fermenter vessel volume, so the productivity effectively depends on the volume of the initial charge to the fermenter. The optimal value is then a compromise between the advantage of leaving a large volume available for addition of fresh nutrient medium against the disadvantage of leaving a large proportion of the fermenter capacity unused for much of the batch cycle (Winkler, 1991). It must also be pointed out that these conclusions are the result of sophisticated mathematical analysis of very simplistic basic equations, which may perhaps be considered as the mathematical analogy of the biblical problem of putting new wine into old wineskins!

The production of glutathione by *S. cerevisiae* KY5711 in fed-batch fermentation was optimized by controlling the specific growth-rate in an optimal profile (Shimizu *et al.*, 1991). The mathematical model was formulated from mass-balances round the fed-batch process and by assuming a relation between the yeast-specific growth-rate and the specific production-rate of glutathione. Mathematical models of heat production by *S. cerevisiae* have been used successfully in the calorimetric control of fed-batch fermentations (Randolph *et al.*, 1990) and in continuous monitoring of glucose consumption, ethanol production and biomass production in batch growth (Larsson, Blomberg and Gustafsson, 1991).

In the growth of recombinant organisms, fed-batch culture has the advantage of enabling the cell-growth phase to be separated from the cloned gene-expression phase, and the effects of different feeding patterns on this separation in a recombinant strain of *S. cerevisiae* have been reported (Patkar and Seo, 1992). In this study, the host strain of *S. cerevisiae* SEY2102 was α haploid, auxotrophic for uracil, leucine and histidine, and could not produce invertase due to a deletion in the chromosomal copy of the *SUC*2 gene. The recombinant strain contained the plasmid pRB58 encompassing the entire yeast *SUC*2 gene, including promoter, signal sequence and structural gene, and also contained the *URA*3 gene complementing the auxotrophic requirement for uracil, and whose protein product thus acts as a marker. High glucose concentrations are known to repress the *SUC*2 promoter. Fed-batch

fermentation allows the system to operate with prolonged periods of low glucose concentration with a suitable glucose feeding strategy. In aerobic closed-batch fermentation, the yeast exhibits diauxic growth. In the fed-batch fermentation, the *SUC2* promoter was derepressed and appeared to respond immediately when the glucose level fell below 2 g litre^{-1}. The specific invertase activity increased continuously as the glucose concentration decreased. This made an interesting comparison with the results of earlier work on the same strain in anaerobic continuous culture, where an optimum glucose concentration was found for maximum invertase activity. The yeast biomass yield coefficient was found to be higher at low glucose concentrations, suggesting a shift to a more energy-efficient pathway. The intention was expressed of determining theoretical optimum glucose feeding profiles on the basis of these experimental results. Yeast cell growth, glucose and oxygen uptake, plasmid segregation and formation of human epidermal growth factor, carbon dioxide and ethanol in recombinant *S. cerevisiae* were successfully predicted for batch, fed-batch and hollow-fibre fermentation by a mathematical model involving 48 parameters, 19 differential equations and 24 analytical equations (Copella and Dhurjati, 1990). Most of the parameters involved were estimated from published data or by regression of experimental data, while eight of the parameters were adjustable.

As one of the most useful applications of fed-batch fermentation is in the utilization of inhibitory substrates, modelling of nutrient feed patterns has received some attention. The most commonly occurring inhibitory effect is where the biomass growth is not subject to inhibition, but product formation is, as in penicillin and lysine production. The least common effect is where both biomass growth and product formation are subject to inhibition. The moderately common situation where fed-batch operation offers the greatest advantage is where product formation is not subject to inhibition, but biomass growth is. In all these situations, it is usually assumed that the computed feeding pattern should first produce optimal biomass growth, and then be altered to give optimal product formation. This involves the implicit assumption that optimally grown biomass is also optimal for product formation. This is particularly relevant in baker's yeast production, as it is widely used as a model optimization system, while there is evidence to suggest that high yeast biomass concentration is associated with low specific invertase activity. The conditions under which yeast is grown are also known to affect the ease of biomass disruption (Wiseman, King and Winkler, 1987), which is relevant to the production of intracellular enzymes such as cytochrome P-450.

As with straightforward fed-batch modelling, the usefulness of the feeding profile depends on the validity of the mathematical expressions describing inhibition. An approach has been developed (Modak and Lim, 1989) in which optimal profiles of medium feed-rate can be generated for growth of single or mixed populations or recombinant organisms in fed-batch fermentation using any number of differential mass-balance equations. The same workers have also developed a procedure for a product-inhibited system, the yeast–ethanol fermentation, using a Michaelis–Menten type of saturation relation to

describe the inhibition effect of ethanol concentration (Modak and Lim, 1987).

CONTINUOUS FERMENTATION

Continuous fermentation has been of interest in yeast fermentations generally because it has the advantages of giving maximum productivity of growth-associated products and of continuous removal of the inhibitory product, ethanol. It should be borne in mind, however, that maximum productivity in continuous systems does not usually coincide with maximum product concentration, which may be more valuable in product recovery — as it is for ethanol production for example. A way round this problem is to couple continuous ethanol production to a pervaporation unit which continuously removes and concentrates the ethanol (Shabtai *et al.*, 1991), or by extractive ethanol recovery from yeast immobilized in a hollow-fibre extractive fermenter (Kang, Shukie and Sirkar, 1990).

Continuous fermentation has long been a favourite of process modellers because this technique allows quasi-steady-state conditions to be achieved. Intracellular metabolite and enzyme assays at steady-state in chemostat cultures of *S. cerevisiae* (Grosz and Stephanopoulos, 1990b) and mathematical models of the associated cellular energetics and catabolism (Grosz and Stephanopoulos, 1990c) have been reported. The influence of dilution rate on synthesis of cloned gene-expression product was investigated in continuous culture of recombinant *S. cerevisiae* producing β-galactosidase (da Silva and Bailey, 1991). This study showed that specific enzyme activity and biomass concentration increased as dilution rate was decreased, and contrary to expectations, overall enzyme productivity was substantially higher at low dilution rate. This suggests that fed-batch fermentation might be the most effective system for utilizing this organism. A strain of *S. cerevisiae* with complete deletion of the chromosomal *SUC* gene, and therefore unable to synthesize invertase, was complemented with the plasmid pRB58 containing the entire yeast *SUC2* gene, including the promoter, signal sequence and structural gene, together with the *URA3* gene, whose protein product acts as a marker (Jang *et al.*, 1990). The growth of this organism was studied in continuous culture, from which an optimal operating strategy for maximum invertase production was deduced. Initially, high cell growth-rate is supported by aerobic growth with high glucose concentrations, followed by anaerobic growth with low residual glucose levels, which again suggests that the process could just as well be carried out in fed-batch or even closed-batch fermentation.

With the advent of sophisticated computer techniques, attention is now being turned to dynamic modelling and quasi-unsteady-state behaviour (O'Neil and Lyberatos, 1990). Several studies of oscillatory behaviour in continuous fermentation of *S. cerevisiae* have been reported. Oscillations have been attributed to low glucose concentrations and moderate dissolved oxygen concentrations, and can be eliminated by either raising or lowering the dissolved oxygen level out of the critical range (Parulekar *et al.*, 1986). Hysteresis, oscillations and maximum specific ethanol productivities in

chemostat culture of *S. cerevisiae* have been reported (Grosz and Stephanopoulos, 1990a), as has the involvement of a cell size control mechanism in the induction and maintenance of oscillations in continuous cultures of budding *S. cerevisiae*, the oscillations occurring spontaneously in glucose-limited culture (Martegani *et al.*, 1990). Investigation of the effect of pH and nitrogen levels on oscillatory behaviour indicated that oscillations are associated with low levels of nitrogen and relatively high pH, while oscillations are suppressed by high ammonium concentration in the feed (Chen, McDonald and Bisson, 1990). Oscillatory behaviour is also associated with partially synchronized cell growth and reproduction at pH 5·5, with a period of 2–3 h (Chen and McDonald, 1990).

CELL-CYCLE MODELLING

In the development of a mathematical model of cytochrome P-450 production by *S. cerevisiae* a promising approach, called *cell-cycle modelling*, is being tried. This has proved successful in generating accurate computer simulations of a hybridoma cell line, mm321 (Faraday and Kirkby, 1992). This generic structured segregated model of the cell cycle is based on the segregation of the cell cycle in terms of biological age, employing a population balance approach which can be solved by Euler integration. While most current models of microbial growth assume an unstructured distributed biophase which does not allow for variations in the biophase, a structured segregated model can allow for changes in cell age distribution. The model segregates the cell population in terms of the phase of cell cycle and the cell age within each phase. Techniques of population balance are then used to generate descriptions of changes in the cell-cycle age distribution. The model can deal with a range of growth conditions, including a variable number of cell-cycle phases, deterministic or stochastic control of phase duration, interactions between phases of the cell cycle, and independent phase interaction with a pre-defined multi-component growth medium. Following its successful application in animal cell culture, it would appear that the cell-cycle model should also be valid for yeast, as a single-celled eukaryote, particularly with the growing importance of yeasts in production of heterologous substances as well as in conventional fermentation products such as ethanol and citric acid.

KNOWLEDGE-BASED CONTROL

Fermentation processes tend to accumulate a folk-lore of empirical and *ad hoc* optimization experience, forming a pool of useful but generally qualitative knowledge which has been difficult to utilize in strictly quantitative computer systems. Methods of making use of such knowledge have been developed, originally for industries such as the cement industry, using 'rule-based' control, and are now being applied to fermentation processes. Reviews of this technique have been presented by Winkler (1990), Hitzmann, Lübbert and Schügerl (1992) and by Konstantinov and Yoshida (1992), who have also developed the *physiological state control* approach to fermentation

modelling which has been summarized by Winkler (1990).

Adaptive control

Clearly the ideal control and optimization system is one which responds to events in the fermentation itself. Pending the development of cell-cycle simulation, the most promising method is *adaptive control*, the name given to the type of control where the controller learns about the process as it controls it. This is a method by which a control model is continually and regularly updated by repeated comparisons with data taken from the process itself, which thus effectively generates its own time-profiles for process control variables. This is based on parameter estimation using on-line mass-balancing. Reviews of the application of adaptive control in biological systems have been produced by Williams (1987a, b) and more briefly by Winkler (1991, 1990, 1988) and Williams (1990).

In a self-tuning or adaptive controller, a *parameter estimator* monitors the process inputs and outputs at pre-set time intervals. It then evaluates the change in values of sets of parameters in a previously defined mathematical model of the process over the duration of the sampling period. From these changes, the parameter estimator computes estimates of the process dynamics in terms of these parameters. A *control design algorithm* then generates controller coefficients from these estimates and the *controller* generates the appropriate control signals to the devices controlling the process. The structuring of the model used by the parameter estimator to compute the estimates of process dynamics is an extremely important feature of an adaptive controller. The process can be represented by a fairly simple set of mass-balance equations, such as those used in optimizing fed-batch fermentations, which may be based on directly measured process parameters, such as pH or exhaust gas carbon dioxide concentration, or indirect parameters calculated from direct parameters, such as biomass concentration.

The application of adaptive control of baker's yeast production has been widely investigated, because the optimal growth conditions can be expressed fairly simply in terms of maintaining a respiration quotient (RQ) at a value of one. Summaries of reports on this work, and also on the application of adaptive control on some systems not involving baker's yeast, including penicillin production, methylotroph biomass production and waste-water treatment, up to 1989 have been reviewed by Winkler (1991). A more recent report illustrated the use of on-line monitoring of fermenter exhaust gas composition in fast inferential adaptive optimization of continuous culture of baker's yeast (Chang and Lim, 1990). This was shown to be several times faster than a system based on measurements of biomass concentration.

Use of genetically engineered yeast to produce foreign (heterologous) cytochromes P-450

The earliest reported success (Oeda, Sakaki and Ohkawa, 1985; Sakaki *et al.*, 1985) in the use of genetically engineered yeast to produce foreign

cytochromes P-450 was the formation of recombinant yeast that expressed rat liver cytochrome P-450c (P-4501A1). A full-length complementary DNA (cDNA), coding for this rat liver 3-methylcholanthrene-induced cytochrome P-450c, was cloned in *Saccharomyces cerevisiae* AM22 cells using an in-house constructed expression vector (based on the plasmid YEp 13) pAMC (made from AAM5; *see* below). The purified enzyme isolated from the microsomal fraction of the mechanically disrupted AM22 yeast cells was shown to be indistinguishable from the enzyme isolated from the rat liver microsomal fraction. Thus the two enzymes could not be distinguished on the basis of apparent molecular weight, chromatographic behaviour, immunochemical properties, spectral properties, or substrate specificity with benzo(a)pyrene and 7-ethoxycoumarin. This rat liver isoenzyme had been fully characterized earlier by these workers (Sakaki *et al.*, 1984). It is of interest that the especially chosen strain of *S. cerevisiae* was grown aerobically in synthetic medium so that Leu+ transformants could be selected for. The medium contained a rather low glucose concentration (2%) and 0·67% yeast nitrogen base, supplemented with amino acids. The pAMC1 vector was constructed from the expression vector pAAM5 by placing the rat cytochrome P-450c complementary DNA, made from isolated messenger RNA using reverse transcriptase, between an alcohol dehydrogenase I promoter and a terminator region. The vector used was readily introduced into the whole cells of *S. cerevisiae* AH22 cells by the use of lithium chloride. The transformation efficiency of this simple technique is similar to that of the methods that employ yeast protoplasts, but without the need to dissolve away the yeast cell wall by using enzymes. It was reported by these authors that the transformed yeast cells produced about 4×10^5 molecules per cell of the cytochrome P-450c, under conditions where apparently no endogenous cytochrome P-450 was made by this strain of yeast, within the limits of spectrophotometric detection by the carbon monoxide binding procedure used. Conditions of glucose repression were probably not reached therefore at 2% glucose concentration with this strain of yeast grown aerobically in synthetic medium. Consideration must always be given to 'plasmid stability' (retention in the yeast cell; or not outgrown by cells containing no plasmid) in the growth procedure for the yeast. The selection of Leu+ mutants, associated with the presence of the plasmid vector, and thus this heterologous cytochrome P-450, is essential for success. Rich media used to grow the brewing strains of yeast such as NCYC 754, as described earlier, cannot be employed in the production of these recombinant yeasts containing foreign cytochromes P-450 because of the absence of direct growth-related selection procedures. Nevertheless, the study of fermentation parameters and use of optimization methods will provide the basis for commercial production of genetically engineered (recombinant) proteins.

Production of animal and human cytochromes P-450 in yeast

Following their success in using genetically engineered yeast to produce foreign cytochromes P-450 (Oeda, Sakaki and Ohkawa, 1985; Sakaki *et al.*,

1985), Sakaki and co-workers have produced many publications on animal cytochromes P-450 expressed in yeast. For example, a chimeric cytochrome P-450 has been constructed from cDNA that expresses cytochromes P-450c and P-450d (Sakaki *et al.*, 1987). Another, very important, construction has used the fused genes for cytochrome P-450c and the NADPH-cytochrome P-450 reductase to demonstrate the increased efficiency of this electron transport chain (Murakami *et al.*, 1987). Further investigations have been reported on the functional analysis of the amino-terminal hydrophobic region and hinge region of this P-450 reductase (Yabusaki *et al.*, 1988). Other studies have involved the expression of bovine enzymes, such as cytochrome p-450c17, progesterone 17α-hydroxylase (Sakaki *et al.*, 1989), and this enzyme fused with the yeast reductase (Shibata *et al.*, 1990). Here, seven fused enzymes were obtained, each differing in the length and amino acid sequence of the hinge region between them. A more recent achievement has been the introduction into yeast of steroid bioconversion steps in a pathway that requires three different cytochromes P-450-catalysed hydroxylation reactions. These steps are catalysed by bovine P-450 17α and by P-450c21 to convert progesterone to 17α-hydroxyprogesterone or 17α-hydroxyprogesterone to 11-deoxycortisol an important intermediate for cortisol production (Ohkawa *et al.*, 1990). A recent review chapter has been published by Yabusaki and Ohkawa (1990) which includes *Escherichia coli*, yeast and mammalian cells in the production of cytochromes P-450.

Other groups have also reported findings on the analysis and exploitation of recombinant cytochromes P-450 in yeast (Kelly and Kelly, 1988), and on the production of cytochromes P-45011β in *Saccharomyces cerevisiae* (Kedzie, Philpot and Halpert, 1991). Other reports have included human cytochrome P-450 expression (Ohgiya *et al.*, 1989) and the expression of a human cytochrome P-4502c gene in yeast, using a galactose-inducible system (Yasumori *et al.*, 1989). The human enzymes have attracted considerable interest, perhaps because of the possible therapeutic use in acute liver failure therapy (or production of known metabolites for comparative studies). Thus the expression of human cytochrome P-4501A1 in *S. cerevisiae* was achieved. Use was made of a 1·70 kb restriction endonuclease fragment containing a full-length human cytochrome P4501A1 cDNA; this was inserted in yeast expression plasmid pMA 91 (with phosphoglycerate kinase promoter) to give the recombinant plasmid pCK-1. Microsomal fraction obtained from the transformed AH22 yeast was shown to metabolize benzo(a)pyrene (Ching *et al.*, 1991). In related work, constitutive and inducible expression of human cytochrome P-4501A1 has been achieved in *S. cerevisiae* (Eugster *et al.*, 1990). Here cDNA was used on a multi-copy plasmid using the acid phosphatase (PHO5) promoter to achieve the inducible expression of the cytochrome P-4501A1. In other recent publications, methods have been described in detail for the expression of mammalian (Güngerich *et al.*, 1991) and human (P-4502c) isoenzyme forms in yeast (Kato, Yasumori and Yamazoe, 1991).

A full scale-up of yeast growth for the application of these achievements will need to be made to produce gram (or even kilogram) quantities of highly

purified human and other cytochromes P-450 for therapy, metabolite production and evolutionary studies.

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