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Transition phase in the production of recombinant proteins in yeast under the *ADH2* promoter: an important step for reproducible manufacturing of a malaria transmission blocking vaccine candidate

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TBV25H, a malaria transmission blocking vaccine candidate, has been cloned in *Saccharomyces cerevisiae* under the control of the glucose repressed *ADH2* promoter. Available fermentation procedures for production of this protein have been unsatisfactory, mainly because of irreproducibility. This work presents an efficient and reproducible method for the production of this vaccine candidate by implementing a three-stage fermentation process. During the first (glucose fed-batch) phase, the promoter is repressed and the culture is allowed to grow exponentially. In the second stage, the glucose supply is provided at a slower constant rate. In the third (ethanol consumption) stage, accumulated ethanol is first allowed to be consumed and an external ethanol supplement is then added as required. The promoter is fully derepressed in this phase, and TBV25H is synthesized. The period of glucose limitation was concluded to be essential for reproducibility. It is presumed that during this period, the culture moves gradually from glucose to ethanol utilization, derepressing the promoter, activating recombinant protein biosynthesis and consequently resuming metabolism without the typical diauxic phase of batch cultures.

Keywords: *ADH2*, TBV25H; *Saccharomyces*; malaria

Introduction

Of the malaria transmission blocking vaccine candidates that are currently available, Pfs25, a sexual stage *Plasmodium falciparum* surface protein, is furthest along in development [12]. Previous attempts to express this protein in *E. coli* resulted in protein that had poor immunogenicity [13]. Immunogenic protein can, however, be obtained from yeast. The protein is currently expressed as a secreted protein, TBV25H, in *Saccharomyces cerevisiae*, under the control of the glucose repressed alcohol dehydrogenase (*ADH2*) promoter [12]. Malaria vaccines are primarily targeted for use in developing countries; therefore there is need for simple production and purification processes that would easily be adapted for use in these countries. In contrast to *E. coli*, there is relatively limited information on recombinant protein expression strategies in yeast, and in particular on the use of the *ADH2* promoter system. We have previously described a fed-batch production scheme based on feeding glucose at a constant rate supplemented by pH-based glucose addition, and followed by ethanol addition [27]. Growth of yeast and expression of TBV25H using this strategy, however, was found to be inconsistent, in part due to variations in the physiological state of yeast cells when the carbon source was switched from glucose to ethanol. In preparation for large-scale GMP production of TBV25H, a more reproducible approach was necessary.

In batch cultures with an excess of glucose, protein expression based on the *ADH2* promoter has three stages. An initial stage where glucose is abundant results in repression of the promoter. In the intermediate stage, low glucose levels allow the promoter to be partially derepressed, and finally, total derepression occurs when the glucose is entirely depleted and an alternate carbon source consumed. Glucose is the preferred carbon source for growth; however, excess glucose is converted by *S. cerevisiae* to ethanol (Crabtree effect) [8,11,19]. While this ethanol can be used as a carbon source for growth, it is less efficient for this purpose than glucose. Because excessive ethanol accumulation is undesirable due to its toxicity, control of glucose levels during the initial growth phase is necessary. As growth is nonlinear, a suitable glucose supply scheme needs to be implemented. A number of control strategies have been devised for minimizing ethanol formation from glucose; the manipulated variable is usually the glucose feed rate [16,21,22]. The glucose feed rate can be correlated to cell mass, or when oxygen is limited, to the oxygen uptake rate. Direct control of glucose using an automated online glucose analyzer is impractical; cell-free broth must be provided to the glucose sensor, but this is difficult at high cell densities where clogging of filters occurs. In addition, conventional glucose electrodes are not autoclavable. An adaptive control technique based on online estimation of ethanol for high cell density fermentations has been reported [10]. This technique accounts for the fact that fed-batch yeast fermentations are nonlinear in nature. However, this control strategy requires the online estimation of specific growth rate from ethanol and exit gas measurements. The simultaneous regulation of both glucose and ethanol using a fuzzy controller has also been reported, and while higher expression of recombinant protein is obtained, the control scheme tends to be complicated [22].

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A convenient and simple approach to controlling the glucose level is its addition in a predetermined exponential manner. This requires, however, that some preliminary experiments be performed to determine the desired glucose supply rate. An alternative to this approach is the use of respiratory quotient (R.Q.) based feedback control. While this necessitates exit gas measurement, this technique is advantageous in that there is no need for preprogrammed feed rates. Also, R.Q. estimation early on tends to be inaccurate due to the low level of biomass present and the small amounts of gases involved. Typically, the culture is allowed to grow in batch mode for a short period before R.Q. based control of the glucose supply rate is started. R.Q. feedback control tends to be oscillatory. In a typical cycle, the control results in an overshoot: excess glucose is pumped into the fermentor with the result that ethanol production occurs. This is followed by a period of glucose exhaustion which can result in partial derepression of the promoter. This is not critical as long as ethanol remains below inhibitory levels and the protein of interest is not toxic, but the R.Q. setpoint used for control needs to be chosen carefully [9]. In practice, R.Q. based control methods require that the R.Q. setpoint be higher than 1.08, to prevent prolonged periods of glucose exhaustion.

The *ADH2* promoter is strongly repressed by glucose [5,18]. However, because glucose has to be tightly controlled to achieve high cell densities with limited ethanol production, promoter derepression can result. This difficulty in controlling the growth and recombinant protein expression phases is evident in the limited availability of literature on the use of the *ADH2* promoter in large-scale systems. While a few approaches are available, they are unsatisfactory because of complexity and lack of reproducibility. For example, recombinant human proinsulin has been produced in high cell density fermentation under the control of the hybrid *ADH2-GAPDH* promoter [25]. The process consists of a batch method to bring the culture to high cell densities followed by a shift in carbon source from glucose to ethanol. The shift was also accompanied by a lowering of temperature from 30°C to 26°C. This process was successful due to either reduced proteolytic activity or improved derepression of the promoter.

Of the four different alcohol dehydrogenase isoenzymes known in yeast, ADHI, ADHIII, and ADHIV are used in the production of ethanol. When glucose is depleted, growth continues on ethanol. with the first step of this process being the conversion of ethanol into acetaldehyde by the ADHII isozyme (Adh2p) [11]. Therefore in the *ADH2* promoter system, the same promoter controls the utilization of ethanol and expression of heterologous protein. A successful transition to growth on ethanol would also result in expression of recombinant protein. A number of regulatory factors are involved in the transition from growth on glucose to growth and expression on ethanol. The regulation of ADHII is controlled by at least two independent mechanisms: a transcriptional activator Adr1p, and the *SNF1* protein kinase [1,24,26]. The Ras/cAMP-signaling pathway is also known to influence gene expression patterns during periods of stress (such as that caused by a change in nutrient) [2,6,20,24].

In this work, we describe the implementation of a three-stage process towards improving the production of the recombinant vaccine candidate. In particular, adaptation of the culture to ethanol metabolism by controlling the glucose level prior to induction seems to improve the reproducibility and extent of induction.

Materials and methods

Strains

Saccharomyces cerevisiae 2905/6 (a/α,*ura3-52,lys2-801,ade2-101,trp1*) contains the plasmid pTBV25H, encoding histidine tagged rPfs25. The plasmid has been described previously [12]. A protease-deficient haploid host, VK1 (2905 (a,*ura3-52,lys2-801,pep4::URA3,trp1*Δ)) was also used to reduce product degradation [3,4].

Starter cultures

The inoculum for fermentor experiments was grown in acid-hydrolyzed casamino acids (20 g L^{-1}), yeast extract (10 g L^{-1}), adenine (400 mg L^{-1}), uracil (400 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (4 g L^{-1}), yeast nitrogen base without amino acids (10 g L^{-1}) and glucose (9%). One hundred milliliter cultures were grown in Tunair baffled shaker flasks (Tunair Inc, Detroit, MI, USA) at 30°C and 250 rpm for up to 16 h. The use of nonselective media has been shown to improve the expression of recombinant protein when under the control of the *ADH2* promoter [18].

Fermentor cultures

Experiments were performed in a bench top fermentor (B Braun Biotech, Allentown, PA, USA) coupled to a data acquisition and control system. Two hundred milliliters of inoculum were added to the fermentor (3 L working volume) prepared with the same medium as above with 4 g L^{-1} glucose. Dissolved oxygen was controlled at 30% of saturation with air, using an adaptive control algorithm [10]. A glucose-rich supplement solution was added to the fermentor based on feedback control of the respiratory quotient (R.Q.). This supplement solution contained 25% glucose, 1% yeast extract, 2% acid-hydrolyzed casamino acids, 1% yeast nitrogen base without amino acids, 400 mg L^{-1} adenine, 400 mg L^{-1} uracil and 4 g L^{-1} $MgSO_4 \cdot 7H_2O$. pH was controlled at 5.0 by the addition of ammonium hydroxide (50% v/v).

After the ethanol produced from the initial growth on glucose phase was almost depleted, an ethanol-rich supplement solution was added to the fermentor (25% ethanol, 1% yeast nitrogen base without amino acids).

Analytical methods

Turbidity was measured at 600 nm using 1× PBS, pH 7.4 for dilutions. Glucose and ethanol in the broth were determined using a glucose analyzer (YSI model 2000, Yellow Springs, OH, USA). Sample analysis was done on a 4–20% SDS-PAGE minigel (Novex, San Diego, CA, USA). The samples were first TCA precipitated (4% deoxycholic acid in 100% trichloroacetic acid) and acetone washed. The gels were stained with Coomassie blue.

Production of TBV25H was followed by determination of total protein binding to nickel affinity resin. Two milliliters of Streamline chelating resin (Amersham Pharmacia

Biotech, Piscataway, NJ, USA) were packed into a 1-cm wide column. The column was washed with water and contacted with 1 ml of 100 mM $NiSO_4$. The column was then washed with 10 ml of 1× PBS, pH 7.4. Ten to 25 ml of broth were clarified by centrifugation at $3000 \times g$ for 20 min and then passed through the column three times. The resin was washed with 20 ml each of 2× PBS, pH 7.4 and 2× PBS, pH 6.4. The bound protein was eluted with 8 ml of 0.25 M sodium acetate, pH 4.5. The eluant was then assayed for total protein using the BCA method. SDS-PAGE analysis of the elutions indicated the presence of nickel-binding impurities.

Results

Two approaches to improving TBV25H biosynthesis were studied. In the first approach the promoter was derepressed either partially or fully throughout the fermentation, by growing *S. cerevisiae* on ethanol or on low levels of glucose. In the second approach, the promoter was fully derepressed only after sufficient biomass was achieved.

a. Ethanol fed-batch fermentation

Expression of TBV25H by the *S. cerevisiae* strain 2905/6, grown on ethanol as the sole carbon source, is shown in Figure 1. After the glucose present in the inoculum (1 g L^{-1}) was consumed, ethanol was supplied initially in batch mode (5 and 10 g L^{-1}) and later as a supplement at a constant rate (15 g L^{-1} h^{-1}), keeping the ethanol concentration below 20 g L^{-1}. R.Q. was stable at ~0.6, consistent with ethanol metabolism. Biomass increased exponentially to a final $OD_{600\ nm}$ value of 190, and 34 mg L^{-1} of TBV25H was obtained after 54 h at an overall specific production rate of 0.0011 mg L^{-1} h^{-1} OD. This strategy is relatively simple, however the fermentation process is prolonged due to the growth rate of yeast being lower on ethanol than glucose.

b. Growth and expression in a glucose fed-batch fermentation

Expression of TBV25H by *S. cerevisiae* 2905/6 grown on glucose as the sole carbon source is shown in Figure 2. After inoculation, the culture was allowed to grow in batch mode until residual glucose was consumed and sufficient biomass had accumulated for glucose supplementation based on R.Q. control at a value of 1.0. This R.Q. setpoint was chosen to ensure a low level of glucose (~2 g L^{-1}) throughout the run. Ethanol accumulation did occur, due to frequent overshoots in glucose addition, but its concentration did not exceed 14 g L^{-1}. For the last 10 h of the process, the ethanol level was fairly stable at 12 g L^{-1}. About half the TBV25H expressed was produced during this period and the specific production rate was also higher, indicating that the *ADH2* promoter was partially derepressed. An $OD_{600\ nm}$ of 160 was obtained, and 60 mg L^{-1} of TBV25H had accumulated after 34 h.

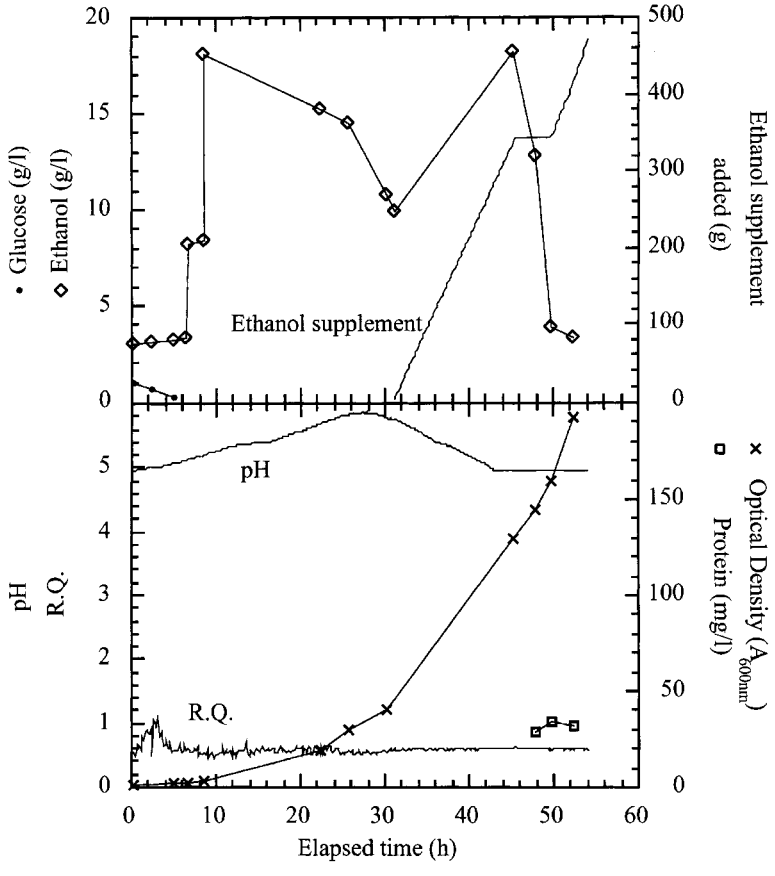

Figure 1 Production of TBV25H from *S. cerevisiae* 2905/6 grown on ethanol as sole carbon source. Glucose (●), ethanol (◇), protein (□), cumulative ethanol supplement, optical density (×), pH and R.Q.

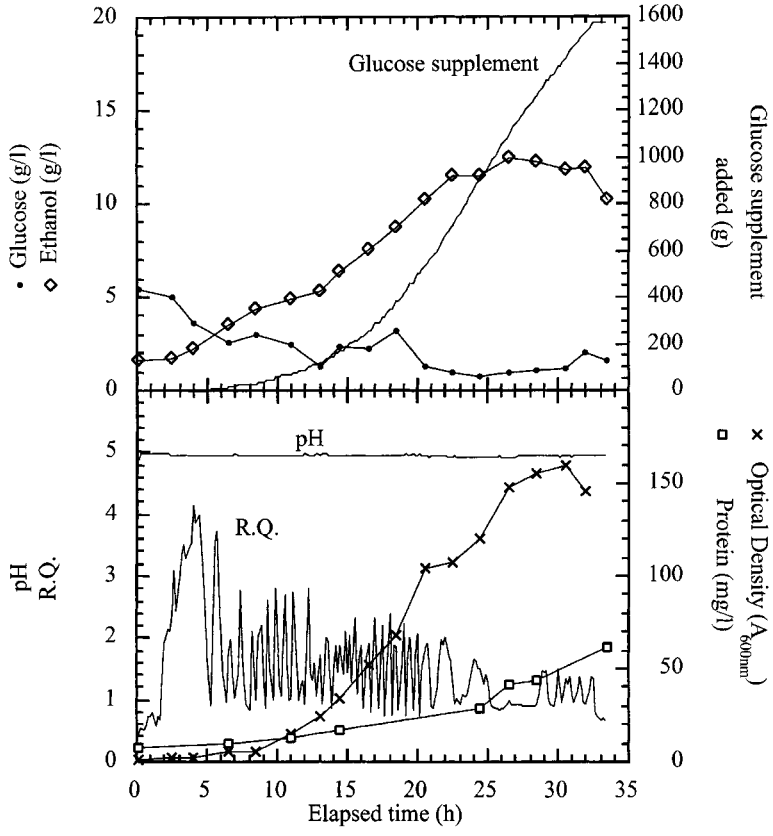

Figure 2 Production of TBV25H from *S. cerevisiae* 2905/6 grown on glucose. Glucose (●), ethanol (◇), cumulative glucose supplement, optical density (×), pH and R.Q.

It was difficult to use the same strategy for the protease-deficient VK1 strain because any excess glucose was rapidly converted to ethanol. Therefore glucose was added in an exponential manner (Figure 3). Ethanol accumulated steadily during the fermentation, but was kept below 15 g L^{-1}. Using this strategy 115 mg L^{-1} of TBV25H and an $OD_{600\,nm}$ of 150 was produced after 26 h.

c. Three-stage fed batch process

In this strategy, the *ADH2* promoter was derepressed only after sufficient biomass had accumulated. Growth and TBV25H expression in 2905/6 is described in Figure 4. In the first stage, glucose was supplied using fed-batch R.Q. control at an R.Q. setpoint of 1.3, thus ensuring the presence of glucose and repression of the promoter. During this period of exponential growth, there was very little production of TBV25H. This stage was followed by a transition period of 2.5 h where the glucose supplement was provided at a limiting constant rate of 8 ml L^{-1} h^{-1}. During this period characterized by an R.Q. of ~1.0, no ethanol accumulation occurred. In the final stage, the supply of glucose was stopped and the cells were allowed to consume accumulated ethanol as carbon source. R.Q. dropped to ~0.6 and a less immediate though pronounced increase in pH was observed. Shortly after, the pH started to decrease and the ethanol level dropped; at this point, ethanol supplement was added to the fermentor at the rate of 12 ml L^{-1} h^{-1}. Final $OD_{600\,nm}$ of 200 and 70 mg L^{-1} of TBV25H were obtained. The highest productivity was achieved during the final stage of the process. The process was terminated when the demand for oxygen levelled off, as determined by the levelling off of agitation and air flow rate profiles (Figure 5).

The behavior of the VK1 strain using this strategy is seen in Figure 6. As indicated in the previous section, feeding of glucose was difficult using R.Q. control, therefore glucose was supplied in an exponential manner. As a result, the ethanol concentration was low and there was no need for a slowdown period. A final $OD_{600\,nm}$ of 130 and 70 mg L^{-1} of TBV25H was obtained.

Discussion

An efficient and reproducible production process has been developed for expression of TBV25H, a recombinant malaria transmission blocking vaccine candidate. The production strategy used previously was based on constant or pH-controlled supply of glucose, followed by fed-batch growth on ethanol. Using this strategy, an average of 30 mg L^{-1} protein was obtained at the end of the process, but the transition from growth to recombinant protein expression was found to be inconsistent. Two major factors account for this inconsistency: an unregulated transition from growth on glucose to induction on ethanol, and ethanol accumulation.

TBV25H expression is growth associated, because the same promoter is responsible for both ethanol utilization and recombinant protein expression. Therefore, it is desir-

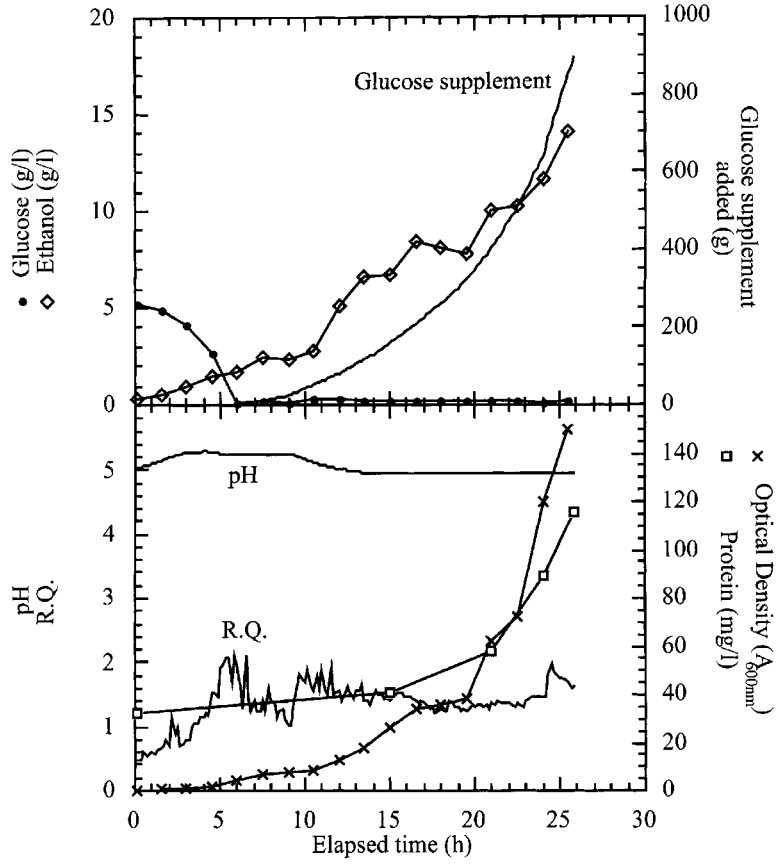

Figure 3 Production of TBV25H from *S. cerevisiae* VK1 grown on glucose. Glucose (●), ethanol (◇), protein (□), cumulative glucose supplement, optical density (×), pH and R.Q.

able to extend the ethanol growth phase for as long as possible. A large-scale production strategy would be based on rapid growth on glucose followed by a glucose depletion phase that derepresses the *ADH2* promoter and activates TBV25H biosynthesis. Growth to high cell densities on glucose requires that ethanol production be minimized; this can be achieved by a fed-batch process where glucose feed rates are steadily increased to match the increasing biomass. Such a fed-batch scheme can be based on R.Q. control or on an exponentially increasing glucose feed rate. Two alternative approaches with the 2905/6 strain were found to be unsatisfactory. In the first, growth and expression on ethanol as sole carbon source required prolonged fermentation. In the second, expression on low levels of glucose was inconsistent.

The transition from growth on glucose to growth and expression on ethanol can be assumed to be the critical stage of this process. If levels of ethanol are already high, it is probable that cells will not respond to a change in carbon metabolism as favorably. Therefore, the growth rate of the cells on glucose (which is already below maximal levels due to the requirement that glucose levels be low), is further reduced by a slowdown in the glucose feed rate. An interesting result of this strategy is that an uninterrupted growth profile is observed, as indicated by biomass production and oxygen consumption (Figure 5). This indicates that the transition period effectively eliminates the diauxic behavior [7,15] normally associated with the shift in metabolism from glucose to ethanol in batch cultures.

Several factors are involved in the regulation of glucose-repressed ADHII [6]. (a) A transcriptional activator (Adr1p), whose translation is controlled by glucose, binds to the upstream activation sequence, UAS1, of the *ADH2* promoter. (b) An independent mechanism involves *SNF1* protein kinase and the *REG1* gene; but the site of action at *ADH2* is currently unknown. (c) A different regulatory mechanism may operate at UAS2, an activation sequence which has been identified just upstream of UAS1. In the presence of glucose, *ADH2* expression is dependent primarily on Adr1p; when derepressed, other factors have been determined to be limiting. In addition, the Ras/cAMP-signaling pathway is known to influence significantly gene expression patterns during shifts in carbon source utilization in batch cultures [2,24]. This pathway is activated by rapidly fermentable sugars like glucose. cAMP has been reported to have a negative effect on *ADH2*; however, while elevated cAMP levels delay the switch to respiratory growth on ethanol, that growth is not prevented [6]. In batch cultures cAMP levels drop as glucose is exhausted and oxidative metabolism of ethanol commences. This reduction in cAMP has been determined to be necessary for managing the diauxic shift from glucose to ethanol [2,20]. Adr1p is a substrate for cyclic AMP-dependent protein kinase (cAPK) *in vitro*, and all the effects of cAPK on

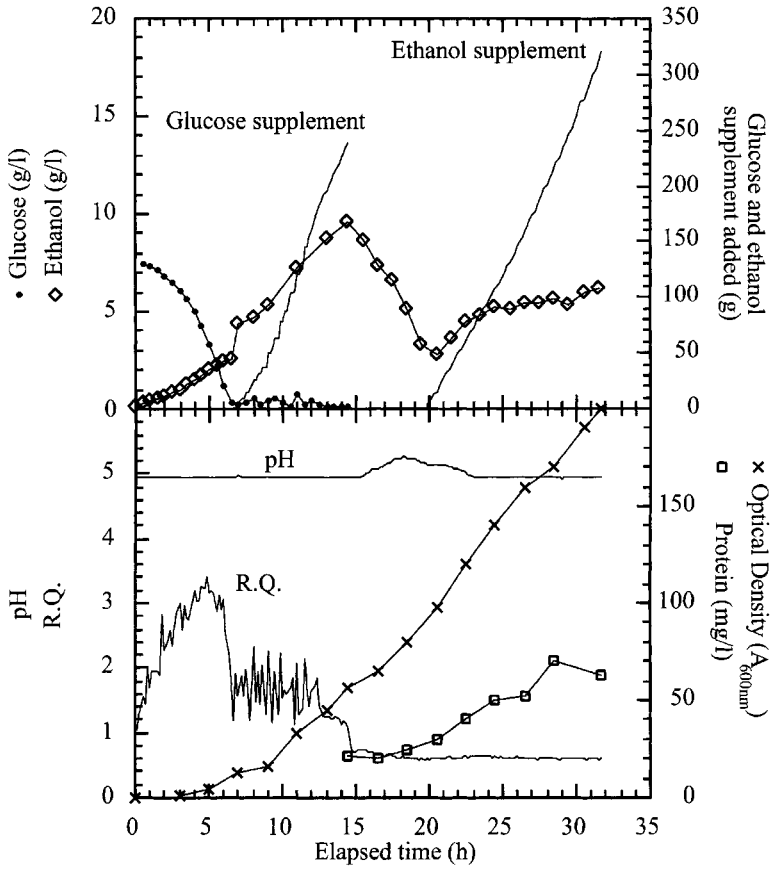

Figure 4 Production of TBV25H from *S. cerevisiae* 2905/6 using a three-stage process. Glucose (●), ethanol (◇), protein (□), cumulative glucose and ethanol supplements, optical density (×), pH and R.Q.

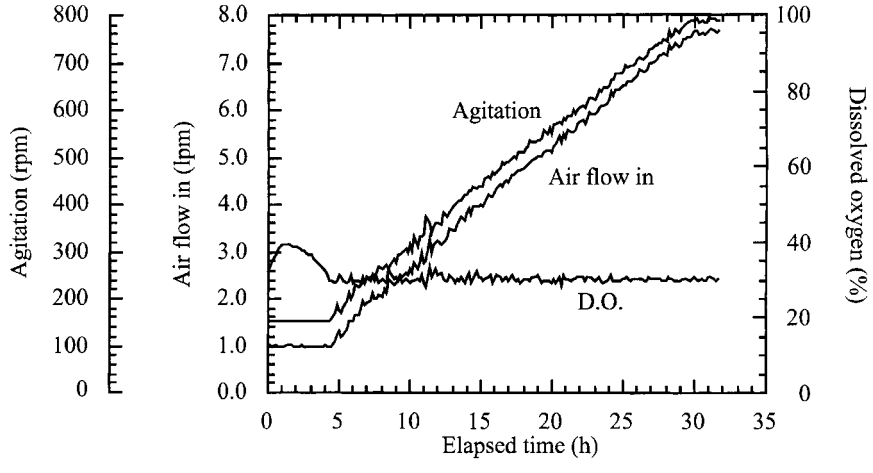

Figure 5 Oxygen utilization of *S. cerevisiae* 2905/6 using a three-stage process.

ADH2 expression are mediated by Adr1p. Since lack of cAPK activity does not result in derepression of *ADH2*, cAPK is not the primary controlling factor in the transition from glucose to ethanol. This corresponds to the view that the Ras-cAMP pathway provides signals for cells to adapt to stress, and is operative as a signaling pathway only during the transition period. The reduction in growth rate, enforced as a slowdown in glucose addition, presumably provides cells with sufficient time to synthesize Adr1p and hence express sufficient amounts of ADHII. In addition, since Adr1p acts as a transcriptional activator of *ADH2*, recombinant protein synthesis is also upregulated.

Similar behavior has been noticed in methylotropic yeasts [23,29]. In the production of aprotinin by *Hansenula polymorpha* under the control of the glycerol repressed *MOX* promoter, a derepression phase is required after high cell densities have been achieved on glycerol [29]. The *MOX* promoter is completely repressed by glucose, and in

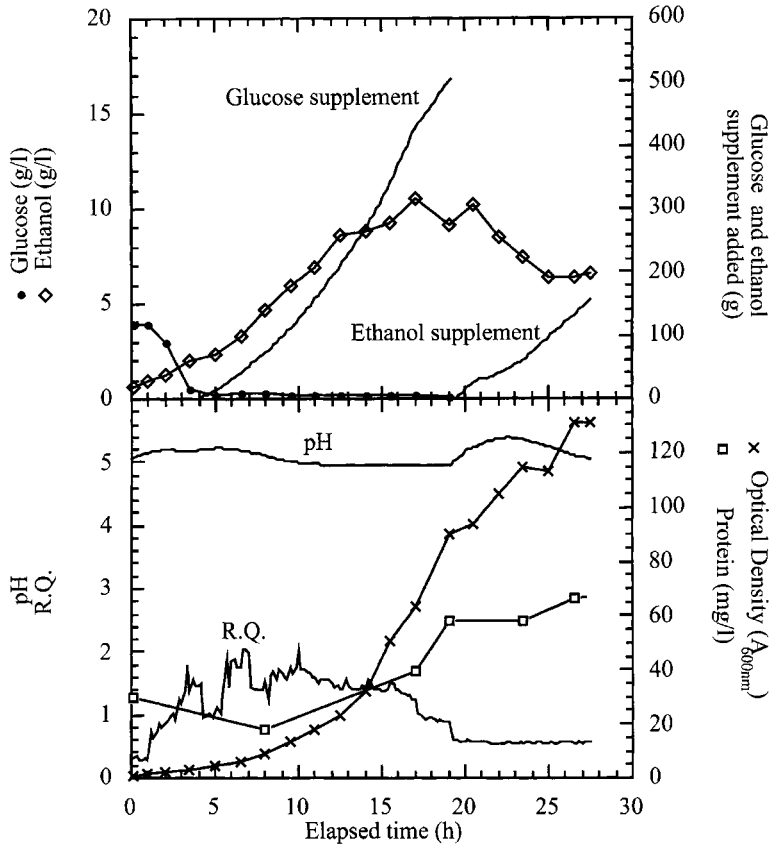

Figure 6 Production of TBV25H from *S. cerevisiae* VK1 using a three-stage process. Glucose (●), ethanol (◇), protein (□), cumulative glucose and ethanol supplements, optical density (×), pH and R.Q.

S. cerevisiae this repression is counteracted by Adr1p [17]. In *Pichia pastoris* recombinant systems, induction of the *AOX1* promoter by methanol can be partially repressed by glycerol, the substrate initially used to obtain high cell densities [23].

The commencement of full derepression of the *ADH2* promoter accompanies the shift in metabolism from glucose to ethanol. In the initial part of this shift, pH increases; the drop in pH that follows coincides with accumulated ethanol dropping to low levels. Addition of external ethanol at the start of the pH increase only delays the stabilization of pH. This pH behavior is standard for yeast cells under the stress of exhaustion of glucose [24,28]. Growth is limited when the ethanol concentration is below 5 g L^{-1} and above 15 g L^{-1} ethanol inhibition is a concern. Therefore the ethanol feed is adjusted frequently to maintain the ethanol level in this range. The feed can be continued till growth stops.

The change in pH observed during the transition can be explained as follows [14,24,28]. During fermentative growth on glucose, intracellular pH is maintained by the plasma membrane H^+-ATPase which excretes protons out of the cytoplasm. Under conditions of stress (eg glucose exhaustion), there is an insufficient supply of ATP; as a result the H^+-ATPase does not pump enough protons out of the cytoplasm, resulting in a lowering of intracellular pH and an increase in extracellular pH. Interestingly, during this transient phase, the Ras-cAMP pathway rapidly improves the availability of ATP, thus restoring intracellular pH. As the culture adapts to respiro-fermentative growth on ethanol, and as the supply of ATP improves, proton excretion out of the cytosol improves, resulting in a drop in extracellular pH. This recovery in extracellular pH serves as a convenient online indicator that the culture has satisfactorily adapted to growth and protein synthesis on ethanol. Furthermore, since the level of ethanol accumulated during growth on glucose is low ($<$20 g L^{-1}), the recovery of pH during the transition phase and early induction usually coincides with the exhaustion of ethanol; this can serve as an indication that ethanol feeding should commence.

When yeast cultures are growing rapidly, usually on an excess of glucose, and when recombinant protein expression depends on a switch of carbon source, sufficient time must be provided for the culture to adapt to the second substrate. If periods of glucose excess exist, with the utilization of a transition period, the culture can always be brought to approximately the same stage/profile of growth, thus allowing for reproducible expression later. This is particularly important in the described system where the same promoter controls both the utilization of the second substrate (ethanol) as well as heterologous protein expression. If the glucose addition mechanism turns out to be self-limiting (eg exponential feed is not fast enough), then the culture will automatically enter a phase where both fermentation and respiration occur simultaneously, and a deliberate slowdown will be unnecessary.

References

1 Boucherie H. 1985. Protein synthesis during transition and stationary phases under glucose limitation in *Saccharomyces cerevisiae*. J Bacteriol 161: 385–392.
2 Boy-Marcotte E, D Tadi, M Perrot, H Boucherie and M Jacquet. 1996. High cAMP levels antagonize the reprogramming of gene expression that occurs at the diauxic shift in *Saccharomyces cerevisiae*. Microbiology 142: 459–467.
3 Chen D, L Chuang, W Chen and T Kuo. 1995. Abnormal growth induced by the expression of HBsAg in the secretion pathway of *S. cerevisiae pep4* mutants. Curr Genet 27: 201–206.
4 Chiang H-L and R Schekman. 1991. Regulated import and degradation of a cytosolic protein in the yeast vacuole. Nature 350: 313–318.
5 Clare JJ and MA Romanos. 1996. Expression of cloned genes in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*. Meth Mol Cell Biol 5: 319–329.
6 Denis CL, SC Fontaine, D Chase, BE Kemp and LT Bemis. 1992. *ADR1^c* mutations enhance the ability of ADR1 to activate transcription by a mechanism that is independent of effects on cyclic AMP-dependent protein kinase phosphorylation of ser-230. Mol Cell Biol 12: 1507–1514.
7 Fiechter A, GF Fuhrmann and O Kappeli. 1981. Regulation of glucose metabolism in growing yeast cells. Adv Microbial Physiol 22: 123–183.
8 Gancedo C and R Serrano. 1989. Energy-yielding metabolism. In: The Yeasts, Vol 3, Metabolism and Physiology of Yeasts (Rose AH and JS Harrison, eds), pp 205–259, Academic Press, London.
9 Hensing MCM, RJ Rouwenhorst, JJ Heijnen, JP van Dijken and JT Pronk. 1995. Physiological and technological aspects of large-scale heterologous-protein production with yeasts. Antonie van Leeuwenhoek 67: 261–279.
10 Hsiao J, M Ahluwahlia, JB Kaufman, TR Clem and J Shiloach. 1992. Adaptive control strategy for maintaining dissolved oxygen concentration in high density growth of recombinant *E. coli*. Ann NY Acad Sci 665: 320–333.
11 Johnston M and M Carlson. 1992. Regulation of carbon and phosphate utilization. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*, Vol 2, Gene Expression (Jones EW, JR Pringle and JR Broach, eds), pp 193–281, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
12 Kaslow DC and J Shiloach. 1994. Production, purification and immunogenicity of a malaria transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified using nickel-NTA agarose. Bio/Technology 12: 494–499.
13 Kaslow DC, IC Bathurst, T Lensen, T Ponnadurai, PJ Barr and DB Keister. 1994. *Saccharomyces cerevisiae* recombinant Pfs25 adsorbed to alum elicits antibodies that block transmission of *Plasmodium falciparum*. Infect Immun 62: 5576–5580.
14 Leao C and N Van Uden. 1984. Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 774: 43–48.
15 Lewis JG, CJ Northcott, RP Learmonth, PV Attfield and K Watson. 1993. The need for consistent nomenclature and assessment of growth phases in diauxic cultures of *Saccharomyces cerevisiae*. J Gen Microbiol 139: 835–839.
16 O'Connor GM, F Sanchez-Riera and CL Cooney. 1992. Design and evaluation of control strategies for high cell density fermentations. Biotechnol Bioeng 39: 293–304.
17 Pereira GG and CP Hollenberg. 1996. Conserved regulation of the *Hansenula polymorpha MOX* promoter in *Saccharomyces cerevisiae* reveals insights in the transcriptional activation by Adr1p. Eur J Biochem 238: 181–191.
18 Price VL, WE Taylor, W Clevenger, M Worthington and ET Young. 1990. Expression of heterologous proteins in *Saccharomyces cerevisiae* using the *ADH2* promoter. Meth Enzymol 185: 308–318.
19 Ratledge C. 1991. Yeast physiology—a micro-synopsis. Bioprocess Engineering 6: 195–203.
20 Russell M, J Bradshaw-Rouse, D Markwardt and W Heideman. 1993. Changes in gene expression in the ras/adenylate cyclase system of *Saccharomyces cerevisiae*: correlation with cAMP levels and growth arrest. Mol Biol Cell 4: 757–765.
21 Shimizu K, M Morikawa, S Mizutani, S Iijima, M Matsubara and T Kobayashi. 1988. Comparison of control techniques for Baker's yeast culture using an automatic glucose analyzer. J Chem Engin Japan 21: 113–117.
22 Shiba S, Y Noshida, YS Park, S Iijima and T Kobayashi. 1994. Improvement of cloned α-amylase gene expression in fed-batch culture of recombinant *Saccharomyces cerevisiae* by regulating both glucose and ethanol concentrations using a fuzzy controller. Biotechnol Bioeng 44: 1055–1063.
23 Sreekrishna K, RG Brankamp, KE Kropp, DT Blankenship, J Tsay, PL Smith, JD Wierschke, A Subramaniam and LA Birkenberger. 1997. Strategies for the optimal production and secretion of heterologous proteins in the methylotropic yeast *Pichia pastoris*. Gene 190: 55–62.
24 Thevelein JM. 1994. Signal transduction in yeast. Yeast 10: 1753–1790.
25 Tøttrup HV and S Carlsen. 1990. A process for the production of human proinsulin in *Saccharomyces cerevisiae*. Biotechnol Bioeng 35: 339–348.
26 Vallari RC, WJ Cook, DC Audino, MJ Morgan, DE Jensen, AP Laudano and CL Denis. 1992. Glucose repression of the yeast *ADH2* gene occurs through multiple mechanisms, including control of the protein synthesis of its transcriptional activator, ADR1. Mol Cell Biol 12: 1663–1673.
27 Wang M-Y, DC Kaslow and J Shiloach. 1996. Production of a malaria transmission-blocking protein from recombinant yeast. Ann NY Acad Sci 782: 123–132.
28 Weitzel G, U Pilatus and L Rensing. 1987. The cytoplasmic pH, ATP content and total protein synthesis rate during heat-shock protein inducing treatments in yeast. Exp Cell Res 170: 64–79.
29 Zurek C, E Kubis, P Keup, D Horlein, J Beunink, J Thommes, M Kula, CP Hollenberg and G Gellissen. 1996. Production of two aprotinin variants in *Hansenula polymorpha*. Proc Biochem 31: 679–689.