

Automated fed-batch culture of recombinant *Saccharomyces cerevisiae* based on on-line monitored maximum substrate uptake rate

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Abstract

A novel method is developed for on-line monitoring of maximum substrate uptake rate (MSUR) for automated control of substrate feeding in aerobic fed-batch cultures. The control scheme depends on real-time measurements of dissolved oxygen (DO) following a process perturbation: the feeding is interrupted momentarily and the instance of complete depletion of substrate is detected by the rise in DO. Immediately, a known amount of substrate is pulsed into the fermentor. The DO level declines, but soon rises (the 'second' rise) as the added pulse is exhausted. The interval between the instances of decline and the 'second' rise of DO, and the known quantity of pulse, are used to compute the MSUR. The feeding then resumes at \leq MSUR. In production of glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) by recombinant *Saccharomyces cerevisiae*, the MSUR-based feeding assured maximum substrate utilization rate, but suppressed formation of ethanol. This approach enabled rapid production of recombinant glucoamylase and enhanced yield. In addition, the extent of substrate limitation could be quantified. A 100% degree of substrate limitation (DSL) was close to optimal for maximizing the enzyme productivity. The MSUR monitoring allowed some starvation of the cells for potential enhancement of the recombinant plasmid stability in certain situations. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Recombinant *Saccharomyces cerevisiae*; Glucoamylase; On-line monitoring and control; Plasmid stability; Fed-batch fermentation

1. Introduction

Baker's yeast, *Saccharomyces cerevisiae*, is an especially attractive alternative host for the expression of recombinant proteins: unlike bacteria such as *Escherichia coli*, the yeast does not produce endotoxins and it performs some post-translational and post-transcriptional modifications that are characteristic of eukaryotes [1]. Moreover, because of a long history of commercial use, substantial information already exists on traditional yeast fermentations [2–4]. Yeast genetics are relatively well known and, from a bioprocessing point of view, extracellular secretion capability of the yeast is an attractive feature. Extracellular secretion of a recombinant protein simplifies recovery and purification [5].

Three major metabolic pathways have been reported for recombinant *S. cerevisiae*: glucose fermentation, glucose oxidation, and ethanol oxidation. Production of recombinant proteins is generally associated only with the oxidative pathways; the glucose oxidation pathway is preferred as being

more efficient. Nonetheless, during production of recombinant proteins, undesirable glucose fermentation to ethanol occurs when glucose concentration exceeds about 0.1 g l^{-1} even under highly aerobic conditions. This is the well-known Crabtree effect [3,4,6]. The Crabtree effect may be suppressed by controlling the glucose level in the fermentation broth. The glucose level may be kept below the Crabtree threshold concentration by fed-batch or continuous culture operation. With recombinant microorganisms, extended continuous culture is troublesome because of plasmid instability issues [7]. Although extensive research has been directed to overcoming plasmid instability in continuous culture of recombinant species [7–11] problems remain especially when non-selective media are employed. Fed-batch culture is, therefore, a preferred methodology for cultivation of recombinant cells. Because of limited duration, fed-batch culture avoids serious plasmid instability, generates a high cell density and maximizes the productivity. Fed-batch culture of *S. cerevisiae* and other yeasts requires an efficient strategy for limiting the concentration of the carbon source: too much limitation may result in undesirable loss in growth rate and productivity.

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The carbon source limitation can be quantified using different criteria. One commonly used criterion is the carbon source concentration itself; however, the carbon source concentration does not reflect the potential ability of the cells to utilize the carbon source. Also, on-line monitoring of very low concentrations of the substrate is difficult and usually unreliable. As an alternative marker of glucose limitation, Konstantinov [12] suggested the use of the 'degree of glucose limitation (DGL)' in mammalian cell culture. The DGL was calculated as the ratio of the current glucose uptake rate to the maximum possible uptake rate. The DGL may range over 0–100%, where 0% DGL corresponds to nil glucose in the culture broth as seen, for example, during the stationary phase after the glucose has been depleted in batch culture. A 100% DGL indicates that the cells are consuming glucose at the maximum possible uptake rate. The DGL is a useful index because it quantifies explicitly the physiological effect of the glucose limitation. Moreover, the accuracy of DGL calculation is not compromised by a low glucose concentration [12]. For calculating the DGL, the maximum glucose uptake rate needs to be measured first. The maximum glucose uptake rate (MSUR) is a measure of the potential glucose consuming ability of the cells; the instantaneous concentration of glucose does not indicate MSUR, and for on-line control of feed rate, the MSUR needs also be determined on-line. Takagi et al. [13] reported on using the maximum glucose uptake rate as a control parameter in fed-batch culture of recombinant *E. coli* in combination with on-line measurement of the biomass by laser turbidometry. On-line measurement of MSUR and its use for controlling the feeding are relatively straightforward in bacterial cultures that consume oxygen rapidly. In contrast, with relatively slow growing yeasts that can potentially switch to anaerobic metabolism and also display diauxic growth patterns, on-line determination of MSUR and its use in controlled feeding remain unproved.

This work demonstrates a novel approach for on-line monitoring of maximum substrate uptake rate (MSUR) in recombinant *S. cerevisiae* culture for producing extracellular glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3). The culture employed the fed-batch methodology and the on-line determined MSUR was used to control the glucose feeding. The effects of the degree of substrate limitation on cell growth, productivity of the recombinant protein, and plasmid stability were investigated.

2. Materials and methods

2.1. Microorganism

The recombinant *S. cerevisiae* C468/pGAC9 (ATCC 20690) was employed [14]. The plasmid pGAC9 coded for glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) which was secreted into the extracellular medium. The plasmid harbored a portion of the yeast 2 μ m plasmid, a DNA fragment from the *E. coli* plasmid pBR322, a DNA fragment

(LEU2 gene) that encoded the LEU2 gene product (leucine), and a section of the glucoamylase gene from *Aspergillus awamori*, under the control of the yeast enolase I promoter and terminator. The host strain C468 was a haploid *S. cerevisiae* with auxotrophic markers for leucine and histidine and carried a mutation (mal) that blocked the utilization of maltose as a carbon source.

2.2. Culture media and inoculum

The recombinant yeast was maintained at 4°C on slants of the YNB selective medium having the following composition: 6.7 g l⁻¹ yeast nitrogen base (without amino acids), 20 g l⁻¹ glucose and 40 mg l⁻¹ histidine [14]. Inoculum was prepared in the selective minimal medium in shake flasks held at 30°C and 180 rpm (Innova 4330 Shaker, New Brunswick Scientific, New Brunswick, NJ, USA) for 24 h. The inoculum size was 10% of the initial volume of the fed-batch broth. A semi-synthetic medium, as detailed previously [2], was used as the initial medium in the fed-batch culture. The feed in the glucose fed-batch culture was a glucose-rich medium that had the same composition as the initial medium except that the glucose concentration was 300 g l⁻¹.

2.3. Culture conditions

Batch and fed-batch cultures were carried out in a bottom-stirred fermentor (MBR Bioreactor, Wetzikon, Switzerland; 15 l nominal, 0.2 m vessel diameter, equipped with two 6-bladed Rushton turbines, 0.07 m in diameter, mounted 0.1 m apart on a central shaft with the lower impeller located 0.06 m above the bottom of the tank; the vessel was fully baffled with four, 0.02 m wide, equally spaced baffles) equipped with controllers for temperature, pH, air flow rate, and agitation speed. All experiments were performed at 30°C and pH 4.5. The latter was controlled by addition of 14.4% ammonia solution as needed. Air flow rate was held at 2 vvm; agitation speed was regulated to maintain the dissolved oxygen concentration above 20% of the air saturation value.

2.4. Instrumentation and computer interface

The software used for on-line monitoring, calculation and control was constructed using the LabVIEW® (National Instruments, TX, USA) graphical programming language installed on a Pentium®-based computer (133 MHz, 32 MB RAM, 1 GB hard disc, Windows® 95). The temperature, agitation rate, pH and DO were monitored and controlled via the process control unit of the MBR fermentor which was interfaced with the computer. The speed controllable feeding pump and the electronic balance for the measurement of feed rate were directly interfaced with the computer. The algorithm for the monitoring of the maximum substrate uptake rate (MSUR) was embedded as a part of the software system (Fig. 1). The on-line, real-time calculation of the MSUR is detailed below.

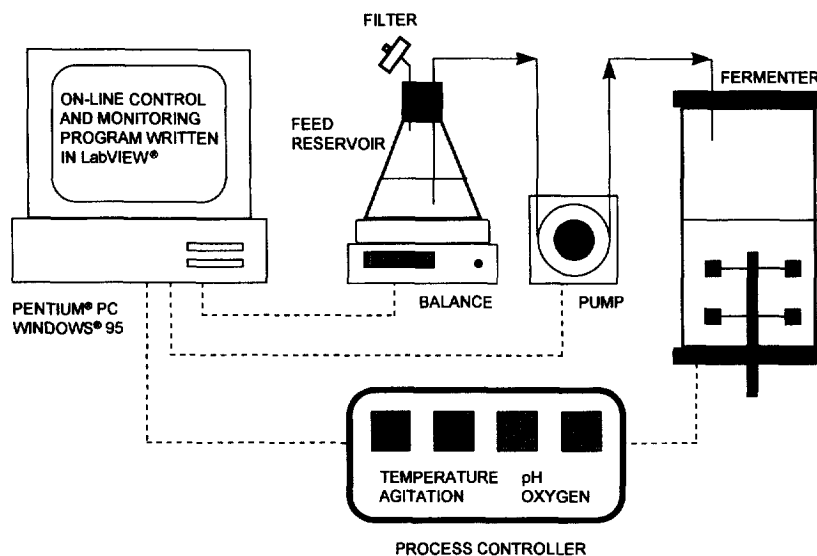


Fig. 1. Schematic representation of instrumentation and software used for automated fed-batch culture system.

2.5. Maximum substrate uptake rate (MSUR) and substrate feed rate control

An on-line perturbation–response analysis procedure was devised to determine the maximum substrate uptake rate (MSUR) and control the feeding. The procedure is explained with reference to Fig. 2 which shows the time profiles of the variables relevant to MSUR determination. The procedure starts with an interruption of the substrate feed flow; the instance of complete depletion of the carbon source (glucose) in the broth is detected by the rapid increase in dissolved oxygen concentration [$t = t_1$ (s)]. As the rise in DO was detected, a certain amount of excess substrate [G (g)] was fed. Because microbial oxidation of the substrate consumed oxygen, the DO concentration declined again and remained at a low value during the period when the carbon source (and hence, oxygen) was being consumed. Upon complete depletion of the carbon source, the DO level rose again [$t = t_2$]. As soon as the second rise was detected, t_2

could be established and the MSUR could be calculated on-line using the equation:

$$\text{MSUR (g h}^{-1}\text{)} = \left(\frac{G}{t_2 - t_1} \right) \times 3600 \quad (1)$$

During the operations for the MSUR calculations, the agitation speed control loop was disabled to prevent an artificial increase in DO. The MSUR measurement procedure was automatically activated every 30 min. Additional details of the MSUR procedure—how the magnitude of the substrate pulse (G) was decided, its effect on the substrate concentration in the fermentor, the length of the feeding period, and the magnitude of the $t_2 - t_1$ relative to the 30 min interval between the MSUR determinations—are noted in Appendix A.

The calculated MSUR was used to establish the subsequent substrate feed rate using the equation:

$$F = f \cdot \text{MSUR} \quad (2)$$

In Eq. (2), the coefficient f was varied in accordance with the desired level of substrate limitation. For 100% degree of substrate limitation, the f -value was unity; whereas for an 80% degree of substrate limitation, the f -value was set to 0.8. The substrate feed rate was automatically updated after each on-line determination of MSUR.

2.6. Analyses

Cell concentration was determined by gravimetry (dry cell weight) and by optical density methods. The gravimetric measurements were in duplicate and each measurement used a 20 ml sample of the broth. The samples were pipetted into two pre-weighed tubes and spun at $250 \times g$, 20-min, under refrigeration (4°C). The precipitates were washed twice by resuspending in 20 ml of deionized water and centrifugally sedimented as described. The washed cells were dried at 100°C for 24 h, allowed to cool in a desiccator, and weighed.

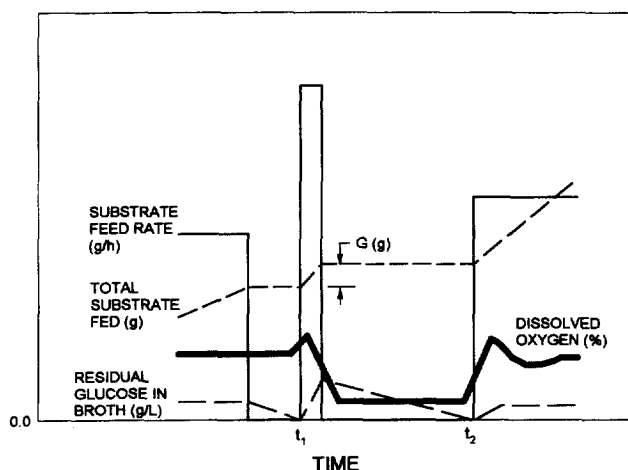


Fig. 2. Time courses of variables related to the maximum substrate uptake rate (MSUR) monitoring.

Optical density (OD) was measured at 600 nm with a spectrophotometer (Pye Unicam SP6-550 UV/VIS, Philips Scientific and Analytical Equipment). The dry cell weight (X) and the optical density (OD) were correlated as follows: $X = 0.4603OD + 0.025$.

Glucose concentration was determined using an enzyme-based kit (Sigma, St. Louis, MO, USA; kit no. 510). The recombinant glucoamylase was assayed by mixing a 0.2 ml aliquot of the cell-free sample with 2% soluble starch (1.8 ml; Sigma S-2630) in 0.1 M citrate buffer followed by incubation at 37°C for 30 min. The reaction was stopped by boiling the reaction mixture for 10 min. The glucose produced during the reaction was assayed using the earlier noted glucose kit. One unit (U) of the glucoamylase activity was defined as the amount of enzyme releasing 1 μmol glucose per minute from soluble starch in 0.1 M citrate buffer (pH 5.0) at 37°C [14]. The ethanol concentration was determined by gas chromatography (Hewlett-Packard 5880A) using PORAPAK-T column (WATERS, Framingham, MA, USA).

The plasmid stability was determined by appropriately diluting (dilution factor: $1 \times 10^{-5} \sim 1 \times 10^{-6}$) the final sample broth of every fermentation run with sterile water and plating 100 μl of the diluted broth on both YNB (selective) and YPG (non-selective) agar plates that were incubated at 30°C for 2 days. All cells could grow on the YPG non-selective plates, but only the plasmid-bearing cells could grow on the YNB selective agar plates. The fraction of plasmid-bearing cells was determined by counting the colonies on the two types of plates (100 ~ 250 colonies per plate). All calculations were based on average counts of three replicate plates. The accuracy and reproducibility of these routine assays have been detailed elsewhere [15].

3. Results and discussion

3.1. Batch culture

Batch culture provided baseline data for comparison with the fed-batch experiments reported later on in this section. Also, even for the fed-batch runs, a batch phase preceded the feeding that began 27 h into fermentations. The batch culture fermentation profiles on the semi-synthetic medium is depicted in Fig. 3. Because of the high initial concentration of glucose, the cells utilized the fermentative pathway for growth, resulting in buildup of ethanol but a relatively slow increase in the biomass level during the first 10 h. Upon complete consumption of glucose, a second growth phase was observed in which accumulated ethanol was utilized as the carbon source. The exponential growth on ethanol was preceded by a second lag phase that commenced at 10 h and continued for a further 5 h (Fig. 3). During this lag period, the biochemical machinery of the cell switched to ethanol utilization from the earlier glucose utilization phase. The culture profile was consistent with diauxic growth on glucose

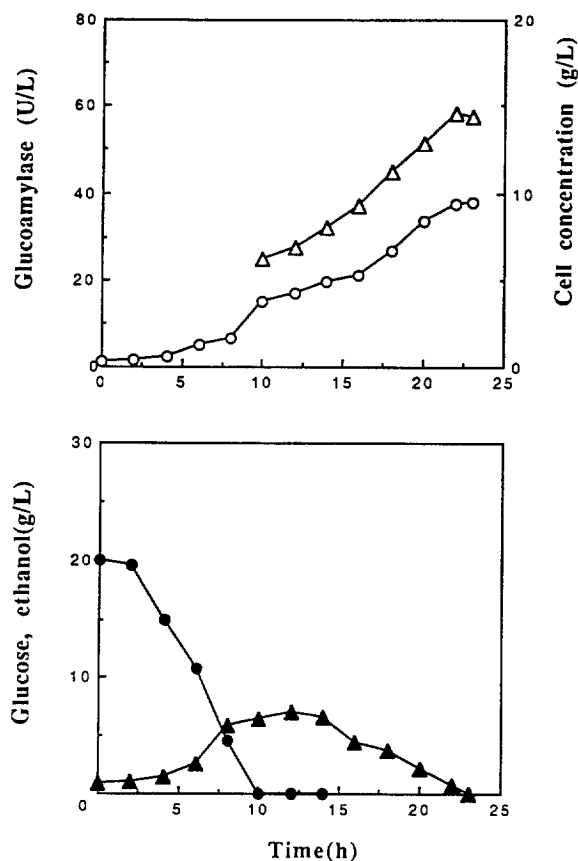


Fig. 3. Time profiles of batch culture with semi-synthetic medium: (○) cell concentration (g l^{-1}); (Δ) glucoamylase (U l^{-1}); (●) glucose (g l^{-1}); (\blacktriangle) ethanol (g l^{-1}).

and ethanol as sequential carbon sources. The specific growth rate on ethanol was much lower than on glucose, and the final biomass concentration was about 10 g l^{-1} .

The final activity of the recombinant glucoamylase was about 60 U l^{-1} (Fig. 3). Glucoamylase measurements were made only upon complete exhaustion of glucose in the culture broth because the glucoamylase assay was based on glucose production by hydrolysis of soluble starch. Most of the glucoamylase was produced during the second phase of diauxic growth when the ethanol accumulated via the preceding fermentative growth was metabolized aerobically.

Oxidation of glucose and ethanol are associated with the tricarboxylic acid (TCA) cycle. The TCA cycle also provides the intermediates for biosynthesis of many amino acids that are used to produce proteins such as glucoamylase. The glucoamylase production profile in Fig. 3 is consistent with growth-associated production which is explained by the link between the TCA cycle and the glucoamylase precursors. Thus, efficient production of the recombinant proteins requires activation of the TCA cycle under aerobic conditions. This is difficult to achieve in batch culture where Crabtree effect cannot be prevented because of a high glucose concentration. Fed-batch cultivation with suitable control of glucose feed rate is known to reduce or eliminate the Crabtree effect [3,4] and improve biomass productivity in culture of

non-recombinant *S. cerevisiae*. A similar effect is expected with recombinant *S. cerevisiae*; hence, fed-batch operation has the potential to enhance productivity of a growth-associated recombinant product.

Significant plasmid loss was not detected during batch culture. The fraction of plasmid-containing cells remained at 75–80% throughout the fermentation. These results agreed with the small-scale batch data reported for the same strain grown on non-selective yeast-extract-peptone-glucose (YPG) medium [15]. Similarly, the plasmid stability results were consistent with reports on production of other recombinant proteins in baker's yeast [6,16].

3.2. Fed-batch culture

3.2.1. Monitoring of MSUR and effects of substrate (glucose) limitation

Fig. 4 shows the results of automated fed-batch culture based on on-line monitored maximum substrate uptake rate (MSUR). The substrate (glucose rich medium) feeding commenced at 27 h after all the carbon source (glucose and ethanol) had been depleted in the batch phase. The feed rate was maintained at the same level as the measured maximum substrate uptake rate (MSUR) ($f=1$ in Eq. (2)), i.e., the

degree of substrate limitation (DSL) was 100%. With this control scheme, the glucose concentration was held at 0.06–0.1 g l⁻¹ and the ethanol level was maintained at 0.6–0.8 g l⁻¹. The biomass concentration reached 22.6 g l⁻¹ at 44 h; the final glucoamylase concentration was 133.7 U l⁻¹. The proportion of the plasmid bearing cells in the final sample was 76%.

The results of more severely limited fed-batch culture (i.e., 80% DSL) are shown in Fig. 5. In this case, the glucose concentration in the broth remained below the detectable level and the ethanol concentration was much lower than in the previous run (Fig. 4). However, the severe glucose limitation had a negative effect on cell growth and enzyme production. The total amount of biomass, the total enzyme production, and the enzyme production rate declined by 16, 13 and 12% (Table 1), respectively, relative to the culture operated at 100% DSL. The biomass yield on glucose was slightly higher in the more severely limited culture (Table 1), suggesting more efficient utilization of glucose for biomass synthesis if glucose conversion to ethanol was suppressed. The plasmid stability (78%) was not significantly affected by changes in DSL. With the MSUR monitoring method, higher than 100% DSL was not practicable. If more

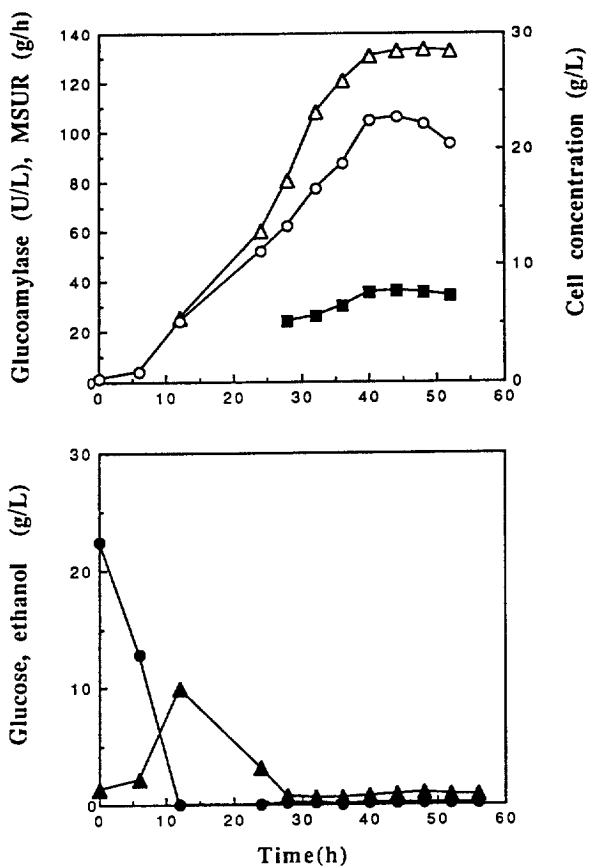


Fig. 4. Time profiles of fed-batch culture with 100% degree of substrate limitation (DSL). The substrate feeding started at 27 h: (○) cell concentration (g l⁻¹); (Δ) glucoamylase (U l⁻¹); (■) MSUR (g h⁻¹); (●) glucose (g l⁻¹); (▲) ethanol (g l⁻¹).

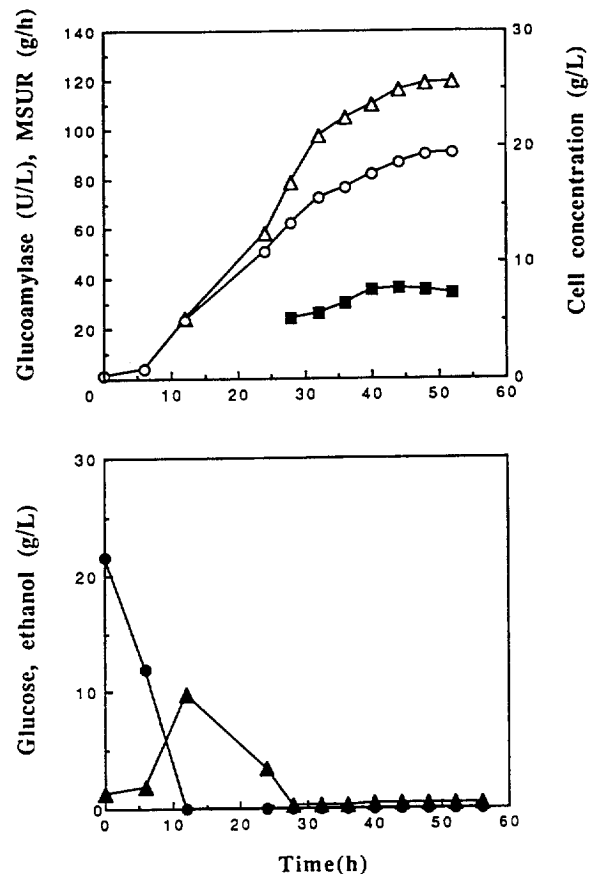


Fig. 5. Time profiles of fed-batch culture with 80% degree of substrate limitation (DSL). The substrate feeding started at 27 h: (○) cell concentration (g l⁻¹); (Δ) glucoamylase (U l⁻¹); (■) MSUR (g h⁻¹); (●) glucose (g l⁻¹); (▲) ethanol (g l⁻¹).

Table 1
Comparisons of yields and productivity

Parameter	Batch culture	Fed-batch (100% DSL ^a)	Fed-batch (80% DSL ^a)
Total glucoamylase production (U)	350.1	893.1	784.8
Total biomass (g, DW)	56.9	137.6	127.2
Specific glucoamylase yield (U g ⁻¹ cell)	6.2	6.5	6.2
Glucoamylase productivity (U h ⁻¹)	14.9	17.2	15.1
Biomass yield based on glucose (g cell g glucose ⁻¹)	0.47	0.41	0.43

^a Degree of substrate limitation.

glucose were fed than the MSUR, glucose would accumulate in the broth and the MSUR monitoring procedure would require a lot more time to attain the instance of glucose depletion.

In the fed-batch experiments reported, the feed rate control based on on-line monitored MSUR could maintain a low glucose concentration (Figs. 4 and 5). Using the MSUR monitoring, the substrate limitation could be quantified, and, hence, the flux of glucose in the metabolic pathway could be controlled effectively. Based on the results, a severe substrate limitation improved efficiency of substrate utilization for biomass synthesis; however, for efficient production of recombinant glucoamylase, the 100% DSL value was closer to the optimal feeding rate trajectory. The 100% DSL operated culture provided the highest specific yield of glucoamylase, the largest quantity of biomass, and the highest production rate of the enzyme (Table 1).

3.2.2. Effect of MSUR monitoring on plasmid stability

Although a non-selective medium was employed, the plasmid stability remained high at 75–80%. A possible explanation for similarly high plasmid stability was advanced by Cheng et al. [17]. Cheng et al. [17] noted that pulsed addition of the growth-limiting substrate (glucose) at suitable intervals with starvation periods between consecutive feeding during fed-batch cultivation had a positive effect on stabilizing the plasmid because the plasmid-free cells lysed or died faster upon depletion of the substrate than did the plasmid-containing cells. As explained in Section 2, during MSUR monitoring procedure, the cells experienced glucose starvation. Even though the starvation periods were very short, they could contribute to improving plasmid stability in situations where no other selection pressure is used and the stability declines with increasing growth rate. Such cases of inverse relationship between plasmid stability and growth rate are common with yeasts [7]. Further work with less stable plasmids is necessary to clarify the impact of MSUR-based controlled cultivation on enhancing plasmid stability.

4. Conclusions

A novel method for on-line monitoring of maximum substrate uptake rate (MSUR) in a microorganism capable of

diauxic growth was developed. The on-line monitored MSUR was used to quantify the degree of substrate limitation (DSL). Moreover, the MSUR was successfully applied to controlling the substrate feed rate in fed-batch culture of recombinant *S. cerevisiae*; hence, enabling efficient production of the recombinant glucoamylase (EC 3.2.1.3). The control was implemented without measuring the substrate on-line. Based on different degrees of substrate limitations in fed-batch culture, a 100% DSL value was close to optimal for attaining maximum productivity. A degree of starvation of the cells during the MSUR monitoring provides an opportunity for improving plasmid stability in situations where stability declines with increasing growth rate. The MSUR-based feeding closely matches the substrate supply to the actual measured demand. The proposed control strategy is potentially suited to aerobic fermentations that exhibit substrate inhibition.

Appendix A

On-line measurement of the maximum substrate uptake rate (MSUR)

The exact volume of broth in the fermentor at any instance could be computed on-line from the known initial volume and the cumulative volume fed up to that time. The cumulative feed volume measurement came from the continuously monitored (Fig. 1) mass of the feed reservoir. The total volume V of the feed pulse could be calculated on-line from the known volume in the fermentor, the known glucose concentration (300 g l^{-1}) in the feed, and the constraint that the glucose concentration in the fermentor at the end of the glucose pulse could not exceed 0.1 g l^{-1} (the Crabtree threshold concentration). For example, for 10 l initial volume in the fermentor, the mass of glucose G needed and the feed pulse volume V could be calculated by simultaneously solving the equations:

$$\frac{G}{10+V} = 0.1 \text{ g l}^{-1}$$

and

$$G = 300 \cdot V$$

Thus, G would amount to 1.0 g and V would compute to 3.3×10^{-3} l.

The calculated volume was fed almost instantaneously (in less than 5 s). As the next rise in dissolved oxygen was detected ($t=t_2$), t_2-t_1 was computed, the MSUR was calculated (Eq. (1)), and the substrate flow resumed at a rate defined by Eq. (2). The actual MSUR calculation from known t_1 , t_2 , and G took less than 5 s; the substrate flow resumed less than 15 s after the second DO rise was detected. The flow was interrupted 30 min later for the next MSUR measurement. The rise in the DO level could be detected about 2–3 min (t_1) after interruption of the feed flow.

The magnitude of t_2-t_1 varied depending on the concentration of biomass in the fermentor; at the maximum substrate uptake rate seen ($\sim 40 \text{ g h}^{-1}$), the 1 g substrate fed to 10 l broth disappeared in 1.5 min. Thus, the interval t_2-t_1 represented 5–10% of the 30 min period between the MSUR determinations.

It is noteworthy that a procedure similar to the one employed for MSUR measurements is commonly used for on-line determination of the volumetric oxygen transfer coefficient: aeration is interrupted briefly and the decline in dissolved oxygen concentration is followed for a few minutes before resuming the air flow [18]. Despite short duration of that measurement and the relatively small changes in the dissolved oxygen level, the signal-to-noise ratio is sufficiently large for reliable dissolved oxygen measurements. Indeed, a high signal-to-noise ratio is reflected in many published data on on-line measured dissolved oxygen concentration during fermentations similar to the one used in this work [3,4].

Appendix

Nomenclature

DGL	Degree of glucose limitation (%)
DO	Dissolved oxygen (%)
DSL	Degree of substrate limitation (%)
DW	Dry weight (g)
F	Substrate feeding rate (g h^{-1})
f	Coefficient in Eq. (2)
G	Added substrate (g)
MSUR	Maximum substrate uptake rate (g h^{-1})
OD	Optical density
t	Time (s)
t_1	Instance of complete depletion of the initial substrate (s)

t_2	Instance of complete depletion of added substrate (s)
TCA	Tricarboxylic acid cycle
V	Volume of the feed pulse needed to provide G g of substrate (l)
X	Dry cell weight (g)

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