Proc. Estonian Acad. Sci. Chem., 1998, **47**, 4, 175–188
https://doi.org/10.3176/chem.1998.4.02

# CONTINUOUS CULTIVATION OF INSECT AND YEAST CELLS AT MAXIMUM SPECIFIC GROWTH RATE

Monika DREWS, Kaja KASEMETS, Ildar NISAMEDTINOV, and Toomas PAALME*

Tallinn Technical University, Ehitajate tee 5, 19086 Tallinn, Estonia and Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 19086 Tallinn, Estonia

Received 24 March 1998, in revised form 11 September 1998

**Abstract.** Two applications of turbidostat culture – the production of recombinant proteins in the insect cell/baculovirus expression system and continuous ethanol production by yeast – are discussed. High specific growth rates 0.036 $h^{-1}$ for insect and 0.33 $h^{-1}$ for yeast cells were maintained in the first stage of the process. The insect cells at maximum metabolic activity resulted in high yields of recombinant proteins: fibrinolytic metalloproteinase from *Vipera lebetina* venom and potato A potyvirus helper component proteinase in the second stage of the process. The yeast cells gave high ethanol yields. The method of steady-state culture with periodic culture takeout was developed and two alternatives, $CO_2$-stat and pH-stat, were analysed in respect of their production of cell material with maximum metabolic activity.

**Key words:** chemostat, turbidostat, pH-auxostat, insect cells, baculovirus expression, distiller's yeast.

## INTRODUCTION

There are two known efficient ways for controlling the cell growth in a culture: cultivation at a desired growth rate and cultivation at a desired cell density or metabolic activity. Cultivation at a desired growth rate can be realized in continuous culture as chemostat [1, 2] or as substrate limited fed-batch culture [3, 4]. The fed-batch method has been demonstrated to be very powerful for the production of yeast starters [5] and recombinant proteins using different host organisms and expression systems, including the insect cell/baculovirus system [6, 7]. However, in some applications, for example with the insect cell/

* Corresponding author, e-mail: tpaalme@kbfi.ee

baculovirus expression system, highly proliferate cells from the exponential growth phase give the best expression of recombinant proteins, and it was suggested [8] that viral multiplication is enhanced because infections occur during the S phase of the cell cycle, leading to rapid development of viral particles and a higher rate of product synthesis.

In the present work, we will show that the methods of continuous cultivation at maximum practical growth rate – modifications of the turbidostat [9] – are very promising for the production of insect cells for baculovirus expression. Another method analysed in this paper is the continuous fermentation of ethanol. In this case, the main reason for cultivating yeast cells at maximum growth rate is to counteract the contamination of the continuous process with lactic acid bacteria, which will significantly reduce the ethanol yield. While in the case of baculovirus expression the process is carried out in absolutely sterile conditions, then in the case of industrial ethanol production, sterilization of all equipment and raw materials would make the process economically less competitive. Therefore, to decrease the energy consumption for both heating and cooling, the mash is heated only up to the temperature optimal for the enzymatic hydrolysis (95 °C), but insufficient to kill all the microorganisms in the grain. An effective way to inhibit the growth of the undesired microorganisms is to decrease pH and the dissolved oxygen concentration down to the level optimal for the selective yeast growth and to maintain the maximum difference of growth rates of the yeast and microorganisms spoiling the process.

The aim of this work was to develop methods of continuous production of cells with maximum metabolic activity and growth rate required for different biotechnological applications. Two very simple methods, one based on controlling the $CO_2$ production rate, originally suggested by Watson in 1969 [10], and the other pH-auxostat [11], in which the biomass concentration is considered to be equal to the pH change during the process, are analysed here in respect of their potential industrial and laboratory applications.

The continuous production of the cells may, however, pose problems in case the cells are required for the simultaneous infection or induction in the second production stage, and any storing of the cells has a clearly negative effect on productivity. In practice, this means that a significant proportion of the culture should be taken out from the fermenter without disturbing the steady state of the rest of the culture. To achieve this goal, we developed the method of steady-state culture with changing culture volume.

## MATERIALS AND METHODS

### Cell lines, viruses

Insect cell line Sf9 (*Spodoptera frugiperda*) adapted for serum-free growth, kindly provided by "KaroBio" (Sweden), was used in the cultivation and

baculovirus expression experiments. Recombinant baculoviruses expressing neurotrophins (NGF, BDNF, NT3, NT4) were prepared in the Institute of Biotechnology of the University of Helsinki by J. Palgi; baculoviruses expressing lebetase, a fibrinolytic metalloproteinase from *Vipera lebetina* venom, and potato A potyvirus helper component proteinase by Dr. Andres Merits (Institute of Chemical Physics and Biophysics, Tallinn). Baculovirus VL-1392-LucGR, expressing beetle luciferase, was obtained from Dr. M. Karp (Centre for Biotechnology, Helsinki). The distiller's yeast strain used in the experiments was kindly provided by MOE Distillery (Tapa, Estonia).

### Culture media

IPL-41 medium (Sigma) with 0.4% yeast extract (Sigma), 0.1% Pluronic-F-68 (Sigma), and antibiotics streptomycin (100 mg $ml^{-1}$, Hybrimax, Sigma) and amphotericine B (1.5 mg $ml^{-1}$, Flow laboratories) was used as a basal medium for the chemostat cultivation and baculovirus expressions. In turbidostat experiments and baculovirus expressions, Insect-Xpress medium (BioWhittaker) was used.

Yeast cells were cultivated on industrial mash containing 130 g glucose $l^{-1}$, pH = 3.8, and on a synthetic medium containing NaCl 4 g $l^{-1}$, $KH_2PO_4$ 1 g $l^{-1}$, $NH_4Cl$ 1 g $l^{-1}$, $MgSO_4 \times 7H_2O$ 0.5 g $l^{-1}$, $CaCl_2 \times 2H_2O$ 33 mg $l^{-1}$, $FeSO_4 \times 7H_2O$ 5 mg $l^{-1}$, $MnSO_4 \times 5H_2O$ 1.6 mg $l^{-1}$, $ZnSO_4 \times 7H_2O$ 1.6 mg $l^{-1}$, $CoCl_2 \times 5H_2O$ 0.3 mg $l^{-1}$, $CuCl_2 \times 5H_2O$ 0.3 mg $l^{-1}$, $Na_2MoO_4 \times H_2O$ 0.3 mg $l^{-1}$, and vitamins myo-inositol 20 mg $l^{-1}$, thiamin 4.4 mg $l^{-1}$, pyridoxine 1.2 mg $l^{-1}$, Ca-pantothenate 0.5 mg $l^{-1}$, and d-biotin 0.03 mg $l^{-1}$.

### Baculovirus expression experiments

Experiments were carried out either on a Braun 1 L fermenter equipped with pH, temperature, and $pO_2$ level control, or in shake flasks with different infection conditions. The infected cultures were sampled during 1–5 days after infection, centrifuged at 1000 rpm and supernatant or cells, depending on the expressed protein, were used for the assays of the recombinant proteins expressed.

### Analytical methods

Cell numbers were counted with a hemocytometer and the viability by trypan blue exclusion. Yeast concentration was determined by measuring the optical density at 540 nm. Sugar and organic acid contents were analysed with HPLC by isocratic elution (0.6 ml $min^{-1}$) of a BioRad HPX-87$H^+$ column with 0.009 N $H_2SO_4$ equipped with $UV_{206}$ and refractive index detectors.

### Cultivation system

The cultivation system consisted of an Applikon 2 L fermenter, controlled through an ADI 1030 biocontroller and AD/DA interface by the BioXpert

("Applikon", The Netherlands) cultivation control program, a commercial version of "FermExpert" [12] developed in our laboratory. The system was equipped with pH, $pO_2$, and temperature sensors and $CO_2$ and oxygen analysers for off-gas analysis. Three variable speed pumps (for feeding the medium, culture takeout, and oxygen control) and one fixed speed pump (alkali) were used by BioXpert control algorithms. The principal control scheme of the cultivation system and control subroutines applied in the different experiments are shown in Fig.1.

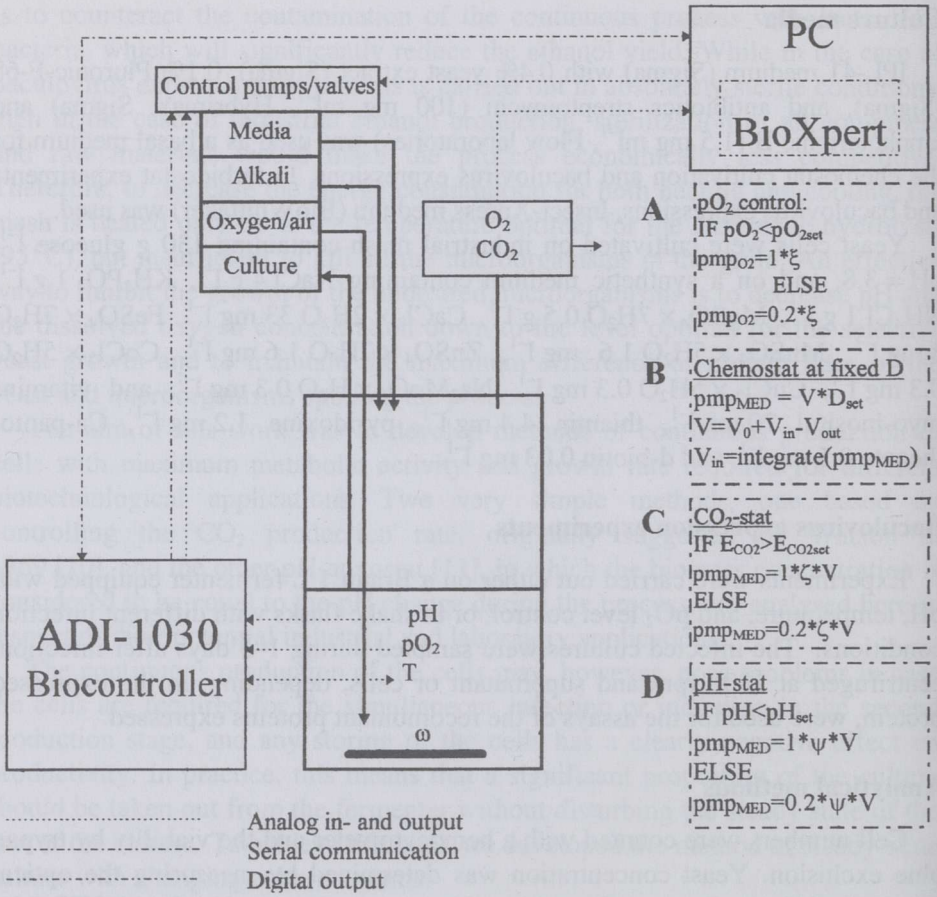



Fig. 1. Control scheme of the cultivation system. $V_0$, initial fermenter volume; $V_{out}$, total amount of culture taken out from the fermenter; $V_{in}$, volume of the cultivation medium pumped into the fermenter; ξ, ψ, ζ, control coefficients; $E_{CO2}$, volumetric $CO_2$ production rate; $E_{CO2set}$, desired volumetric $CO_2$ production rate; $T$, pH, $pO_2$, ω, online measured values of the temperature, pH, dissolved oxygen concentration, and stirrer speed in the culture medium; $O_2$, $CO_2$, online values of oxygen and $CO_2$ concentration in the exhaust gas; $pmp_{MED}$, feeding rate of medium; $pmp_{O2}$, feeding rate of oxygen.

### Cultivation of insect cells in chemostat

The dissolved oxygen level in the fermenter was maintained at 50% of air saturation by introducing pure oxygen into the culture medium with the oxygen pump controlled by BioXpert using control subroutine **A** (Fig. 1). Air was introduced (at a constant rate) into the upper part of the fermenter to facilitate $CO_2$ evolution. The pH level was kept constant at 6.2 using titration with 2N $Na_2CO_3$. The dilution rate ($D$ = medium/$V$) was controlled at the desired level by (i) keeping the culture volume constant by means of an overflow tube or (ii) using algorithm **B** (Fig. 1), in which the value of $V_{out}$ was calculated as:

$$V_{out} = V_0 + V_{in} - V_{real},$$

where $V_{real}$ designates the culture volume measured after culture takeout, $V_{in}$ is the total volume of the medium pumped into the fermenter, and $V_0$ is the initial amount of the culture in the fermenter.

### Turbidostat cultivation

The experimental set-up was the same as for the chemostat cultivation except that (i) autonomous pH control was not used and (ii) in anaerobic conditions neither oxygen nor air was introduced into the fermenter. In $CO_2$-stat experiments the volumetric $CO_2$ production rate, $E_{CO2} = CO_2/V$, was calculated and used for process control according to subroutine **C**. In pH-stat experiments the cultivation process was controlled by subroutine **D** (see Fig. 1).

## RESULTS

### Continuous cultivations of Sf9 cells for baculovirus expression

### Chemostat cultivation with continuous outflow of the culture for baculovirus expression

In the case of the classical chemostat culture, the outflow from the mother fermenter (chemostat) was pumped into the second fermenter (batch), already containing fresh medium and baculovirus. Pumping was stopped after a volume equal to the fresh medium volume in the second fermenter had been added. The expression level of the recombinant neurotrophine BDNF was monitored during the next five days. The maximum expression level, slightly higher than that in the conventional batch procedure, was obtained on the third day of infection.

High expression levels of the recombinant luciferase (175 mg recombinant luciferase $ml^{-1}$) were also obtained if a small portion of high density chemostat culture of insect cells ($10^7$ cells $ml^{-1}$) was diluted five-fold and infected at MOI (multiplicity of infection) three with baculovirus [13].

## Chemostat cultivation with periodic takeout of the culture for baculovirus expression

We studied the cultivation of the insect cells in steady-state culture (chemostat) in conditions of takeout of about half of the culture every day for baculovirus expression considering that the simultaneous infection of the cells results in higher expression yields of the recombinant proteins. The subroutine **B** (Fig. 1) was used to control the chemostat culture with periodic culture takeout. The dilution rate $D$ corresponding to the growth rate $\mu$ shows that the steady-state insect cell culture was obtained and maintained over a period of one week (Fig. 2).

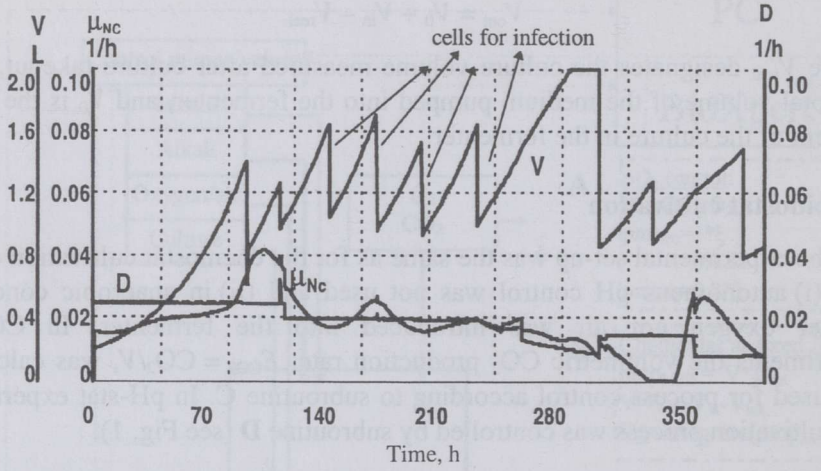



Fig. 2. Chemostat cultivation of insect cell line Sf9 with periodic takeout of cells for baculovirus expression. $V$, culture volume; $D$, dilution rate; $\mu_{NC}$, specific growth rate.

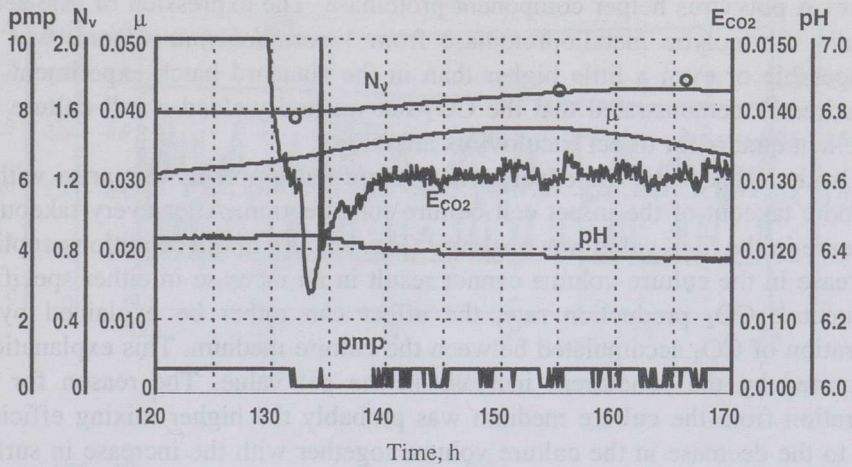
Baculovirus expressions were carried out every day with the cells derived from chemostat by diluting the culture with an equal volume of fresh medium and by infecting batches with baculovirus (MOI = 0.1). Different recombinant proteins luciferase, BDNF, NT3, NT4, and NGF, were expressed in turn. Results demonstrated that the cells derived from chemostat and diluted twofold with fresh cultivation medium gave low (compared to the standard batch experiment) expression levels of all the recombinant proteins listed above.

To obtain from the continuous process cells for baculovirus expression with similar properties to those in industrial batch processes (exponentially growing cells at densities 1–2 million cells $ml^{-1}$) repeatedly, we used the method of turbidostat cultivation.

## Turbidostat culture of insect cells

The culture was obtained by keeping the biomass concentration constant, achieved by controlling $CO_2$ production rate, $E_{CO2}$, at a level corresponding to a cell number of about 1.5 million cells $ml^{-1}$.

(a)




(b)

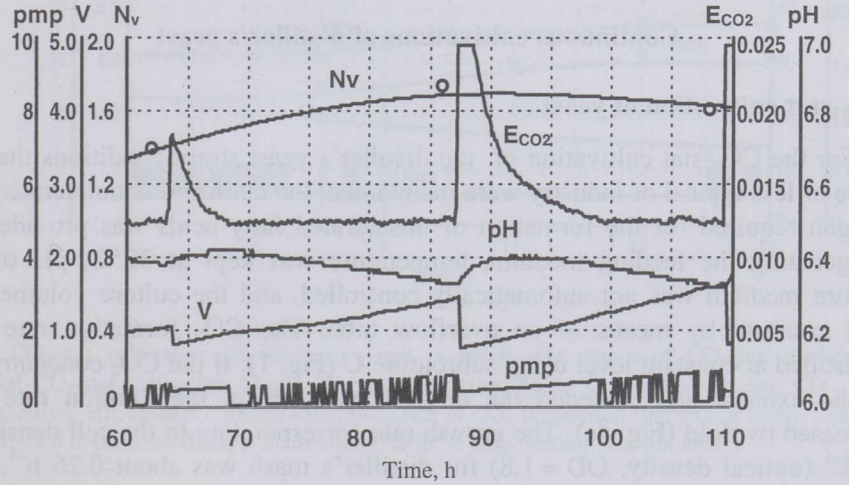



Fig. 3. $CO_2$-stat cultivation of insect cells. a, cultivation at a constant culture volume; b, cultivation with the periodic culture takeout. $N_v$, viable cell count; $E_{CO2}$, volumetric $CO_2$ production rate; $V$, culture volume (1); *pmp*, feeding rate (ml $min^{-1}$).

The results show that by using the $CO_2$-stat (subroutine **C** in Fig. 1) the biomass concentration was kept at a level of 1.5 million cells $ml^{-1}$ for optimal infection (Fig. 3). The viability of the cells obtained by this method was close to 100%. The growth rate $\mu = 0.036\ h^{-1}$ was close to the maximum values reported in the literature for insect cell culture [14, 15]. The culture obtained by this method had an excellent baculovirus expression performance, resulting in the

maximum expression level (more than 10% of the whole cell protein) for a potato A potyvirus helper component proteinase. The expression of the second protein, fibrinolytic metalloproteinase from *Vipera lebetina* venom, was also comparable or even a little higher than in the standard batch experiment. The experiments demonstrated that the $CO_2$-stat method yielded a cell culture with excellent quality for direct baculovirus infection.

On the other hand, the experiment showed the problems that arise with the periodic takeout of the insect cell culture for infection. After every takeout, an increase in the $E_{CO2}$ value was observed (Fig. 3b). As in the conditions applied a decrease in the culture volume cannot result in an increase in either specific or volumetric $CO_2$ production rate, the effect can rather be explained by the liberation of $CO_2$ accumulated between the culture medium. This explanation is supported by the concurrent increase in the pH value. The reason for $CO_2$ liberation from the culture medium was probably the higher mixing efficiency due to the decrease in the culture volume together with the increase in surface-to-volume ratio.

## Continuous cultivations of distiller's yeast

### $CO_2$-stat cultivation of yeast

For the $CO_2$-stat cultivation of the distiller's yeast strain conditions that are more or less typical of industry were maintained: the culture was not aerated, the oxygen required for the formation of unsaturated fatty acids was provided by oxygenating the feeding medium, temperature was kept at 32 °C, pH of the culture medium was not automatically controlled, and the culture volume was kept constant by means of an overflow tube. The $CO_2$ formation rate was controlled at constant level using subroutine **C** (Fig. 1). If the $CO_2$ concentration in the exhaust gas exceeded the desired level SETp, the dilution rate was increased twofold (Fig. 4a). The growth rate corresponding to the cell density of 1 g $l^{-1}$ (optical density, OD = 1.8) for distiller's mash was about 0.26 $h^{-1}$. The maximum ethanol concentration was about 5 g $l^{-1}$.

To study the effect of cell density on $CO_2$ production capacity, the overflow of the $CO_2$-stat culture was pumped during 1 h into a 300 ml fermentation vessel and tested for $CO_2$ production capacity by measuring the $CO_2$ evolution rate for the first 12 h ($Fe_{12}$). The most active cultures were derived at the lowest cell densities (see Fig. 4b) at which the culture also had the highest growth rate of about 0.3 $h^{-1}$ (not shown). The effect of cell density on the growth rate was confirmed by pH-stat experiments, in which a defined mineral medium was used to prevent the possibility of limitation by some of the substrates present in the distiller's mash.

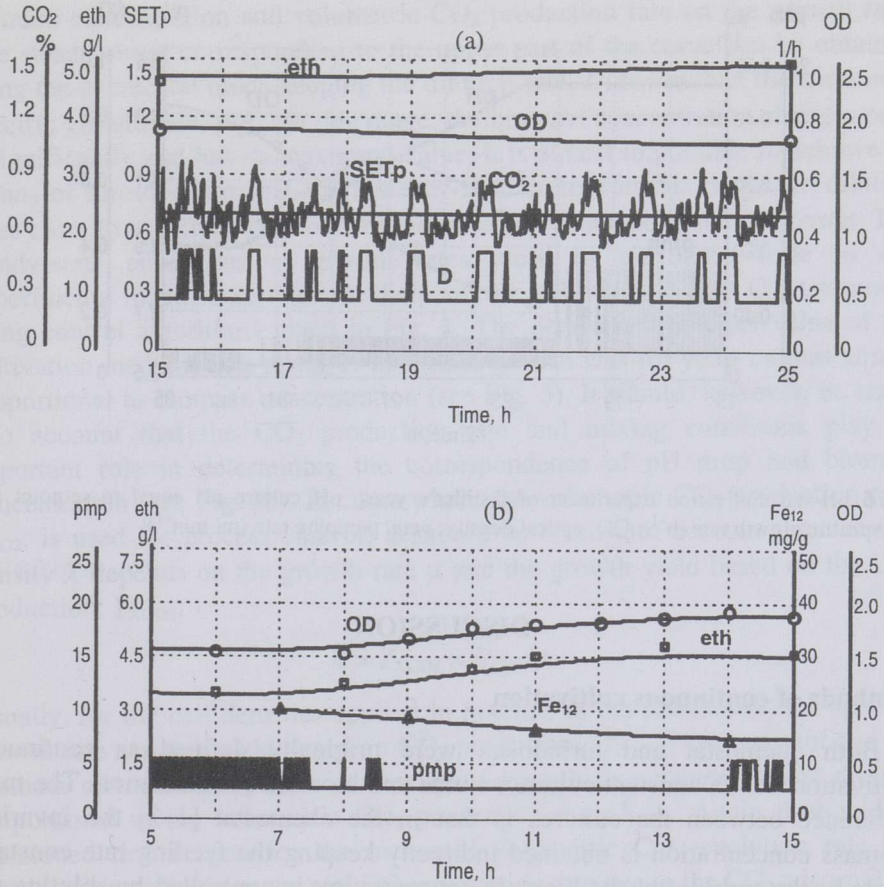



Fig. 4. $CO_2$-stat cultivation of distiller's yeast. a, steady-state phase of $CO_2$-stat cultivation; b, initial $CO_2$-stat cultivation phase. OD, optical density; SETp, $CO_2$ setpoint value; $CO_2$, $CO_2$ concentration; eth, ethanol concentration; $Fe_{12}$, rate of $CO_2$ production (mg $CO_2$ g $mash^{-1}$) during the first 12 h in the second stage; *D*, dilution rate ($h^{-1}$); *pmp*, feeding rate (ml $min^{-1}$).

## pH-stat cultivation of distiller's yeast

The steady-state culture of distiller's yeast was obtained using the control algorithm **D** (Fig. 1). The pH value equal to 3.8 was chosen for the feeding medium to obtain an initial pH setpoint value, $pH_{set}$, of 3.4 for a low initial cell density of about 0.1 g $l^{-1}$ (OD = 0.2). With the decrease of the $pH_{set}$ value the biomass concentration increased almost proportionally, showing that the method can be applied for cultivation at a desired cell density. The specific growth rate, $\mu_X$, increased with increasing cell density up to a value of 0.33 $h^{-1}$ and started to decrease at higher cell densities. The cell density corresponding to the maximum growth rate was 0.2–0.3 g dwt $l^{-1}$ (Fig. 5).

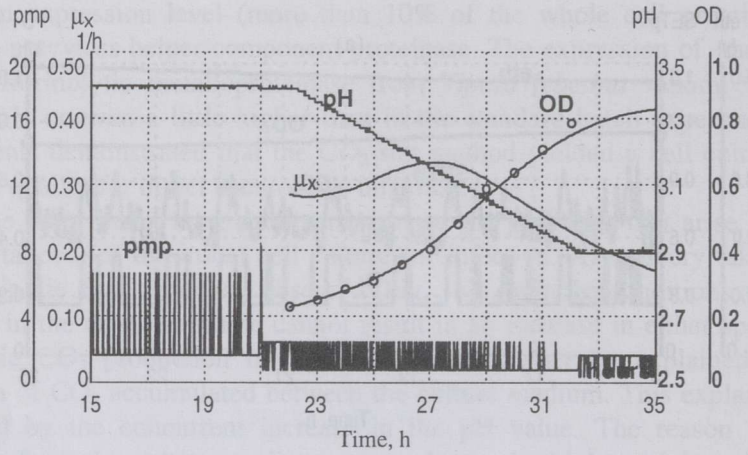



Fig. 5. pH-stat cultivation experiment of distiller's yeast. pH, culture pH equal to setpoint pH; $\mu_X$, specific growth rate ($h^{-1}$); OD, optical density; *pmp*, pumping rate (ml $min^{-1}$).

## DISCUSSION

### Methods of continuous cultivation

Both chemostat and turbidostat were originally defined as continuous cultivation with a constant culture volume and biomass concentration. The main difference between the cultures is that in the chemostat [1, 2] the invariant biomass concentration is obtained indirectly keeping the feeding rate constant, while in the turbidostat the biomass concentration is controlled by diluting the culture with a fresh cultivation medium [9]. Traditionally for both, the culture volume is kept constant using an overflow tube. In the chemostat, steady-state (invariant biomass concentration during the time span of observation) is achieved by setting the dilution rate (feeding rate per unit of culture volume) constant and lower than the maximum of the specific growth rate. In the turbidostat, the biomass concentration is kept constant by means of a feed-back control loop starting feeding at $D > \mu_{max}$ at cell densities exceeding the desired level. Originally, the biomass determination was based on the measurement of the turbidity or the optical density of the culture. Unfortunately, the applications of the method are limited to the clear culture media and low cell densities because of the danger of wall growth and are therefore infrequently used in practice. Less direct methods of growth control were developed based on the measurement of the pH difference of the culture and the feeding medium [11], oxygen consumption [16], and $CO_2$ production rate [10]. The volumetric oxygen consumption rate, $E_{O2}$, and volumetric $CO_2$ production rate, $E_{CO2}$ ($CO_2$ production rate per culture volume), are at maximum growth rates proportional to biomass concentration. Figure 6 illustrates the proposed dependence of the

biomass concentration and volumetric $CO_2$ production rate on the growth rate. The steady-states corresponding to the upper part of the curve can be obtained using the chemostat mode keeping the dilution rate, $D$, lower than the maximum specific growth rate, $\mu_{max.}$. In this range, the biomass concentration changes only insignificantly and has its maximum value. It is almost impossible to achieve by means of turbidostat or pH-stat a steady-state culture in this range. At dilution rates close to $\mu_{max}$ the "wash out" of the culture is observed in the chemostat. The steady-state conditions at growth rates close to maximum were in our experiments maintained with the help of the pH-auxostat or $CO_2$-stat modes using control algorithms given in Fig. 1. The decrease in the pH value of the cultivation medium as a result of microbial growth was for yeast culture almost proportional to biomass concentration (see Fig. 5). It should, however, be taken into account that the $CO_2$ production rate and mixing conditions play an important role in determining the correspondence of pH drop and biomass concentration (see Fig. 3b). In cases where the volumetric $CO_2$ production rate, $E_{CO2}$, is used for process control, it should be taken into account that the cell density $X$ depends on the growth rate $\mu$ and the growth yield based on the $CO_2$ production, $Y_{XCO2}$:

$$X = Y_{XCO2} \times E_{CO2}/\mu \, .$$

Usually, for the cell densities applied in practice in the turbidostat, the specific growth rate and cell yield based on $CO_2$ production ($Y_{XCO2}$) are constant and the biomass concentration, $X$, is at maximum growth rate values almost directly proportional to the volumetric $CO_2$ production rate, $E_{CO2}$. Again it should be mentioned that in practice the measured volumetric $CO_2$ evolution rate can considerably differ from the real $CO_2$ production rate due to the $CO_2$ adsorption/ desorption processes.

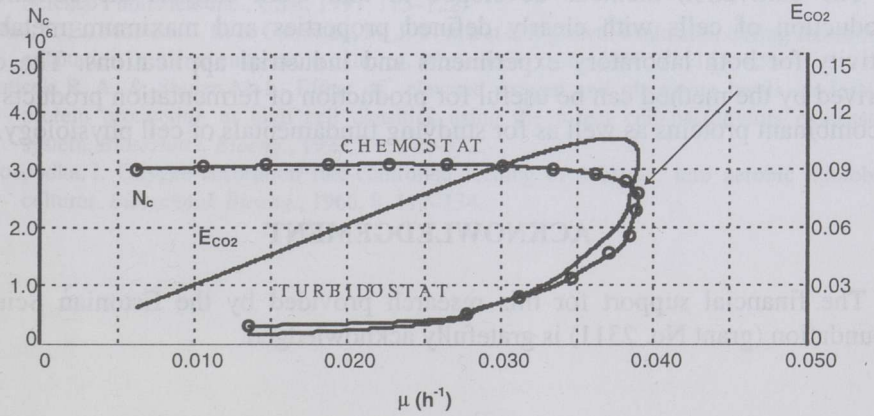



Fig. 6. Proposed dependence of the cell density, $N_c$, and specific $CO_2$ production rate on the specific growth rate, $\mu$. $E_{CO2}$, volumetric $CO_2$ production rate.

Because of the effect of autocrine factors or substrate inhibition two steady-state values can correspond to the same volumetric $CO_2$ production rate and biomass density (Fig. 6). The steady-state obtained depends on the control algorithm. Using the traditional mode, in which the pump is switched on at $E_{CO2} > E_{CO2set}$ or $N_c > N_{cset}$, we can obtain the steady-states corresponding to the lower part of the curve. Switching the pump off at volumetric $CO_2$ production rates exceeding the setpoint value, we can obtain the steady-states similar to the chemostat.

Our results demonstrated that combining very simple control subroutines (**A–D**), cultures of insect and yeast cells can be grown at growth rates maximum for current cultivation conditions using either $CO_2$-stat or pH-stat. In laboratory conditions, the pH-stat method seemed to be more precise and reliable than $CO_2$-stat, at least for yeast cells. On the other hand, in industrial conditions pH and the composition of the feeding medium (and buffering capacity) can vary significantly and result in significant variation of the desired biomass concentration if pH-stat is applied. Another factor that can affect pH-stat in industry is inhomogeneity of the culture in big fermenters commonly used in distilleries. Therefore, pH-auxostat, which is superior in laboratory conditions together with its simplicity in realization, may not be so effective in industrial conditions. As the $CO_2$-stat cultivation data show (see Fig. 3b), the exact maintenance of the setpoint value is not very critical to the process in the long term, especially if the upper dilution rate limit is chosen to be only a little higher than the predicted maximum of the specific growth rate. The lower pumping range was varied in our experiments, but was never chosen to be less than 20% of the maximum growth rate. This is especially important in case of industrial culture media, which are often not completely balanced for economical reasons. If pulsing of the feeding rate irreversibly affects culture activity, special controllers can be built in; however, no practical need to use them can be inferred from our experiments.

The cultivation methods developed in this work support the continuous production of cells with clearly defined properties and maximum metabolic activity for both laboratory experiments and industrial applications. The cells derived by the method can be useful for production of fermentation products and recombinant proteins as well as for studying fundamentals of cell physiology.

## ACKNOWLEDGEMENT

The financial support for this research provided by the Estonian Science Foundation (grant No. 2311) is gratefully acknowledged.

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# PUTUKA- JA PÄRMIRAKKUDE PIDEVKULTIVEERIMINE MAKSIMAALSEL KASVUKIIRUSEL

Monika DREWS, Kaja KASEMETS, Ildar NISAMEDTINOV
ja Toomas PAALME

Kemostaatne kultiveerimismeetod on levinuim nii laboratoorsel kui ka tööstuslikul pidevkultiveerimisel. Samal ajal on harva kasutataval turbostaadil (seade võimaldab kultiveerimist soovitud rakutihedusel ja maksimaalsel kasvukiirusel) mitmeid eeliseid mõnede laboratoorsete rakenduste ja tööstuslike protsesside puhul. Siinses uurimuses on analüüsitud kahte võimalikku turbostaadi rakendusala: rekombinantsete valkude tootmist putukarakkude ja bakuloviiruse ekspressioonisüsteemis ja etanooli pidevtootmist pärmirakkude abil. Protsessi esimeses staadiumis saavutati putukarakkude ja pärmi suur erikasvukiirus, vastavalt 0,036 ja 0,33 $h^{-1}$. Saadud metaboolselt aktiivsed putukarakud andsid infektsioonil rekombinantsete bakuloviirustega kõrge võõrvalkude ekspressiooni ning pärmikultuuri etanoolisaagis oli suur. On välja töötatud muutuva mahuga *steady-state*'i kultiveerimismeetod ja analüüsitud võrdlevalt kahe alternatiivse võimaluse – $CO_2$- ja pH-staadi – rakendamist maksimaalse metaboolse aktiivsusega rakumaterjali saamisel.