Modification of A-stat for the characterization of microorganisms

Kaja Kasemets\textsuperscript{a}, Monika Drews\textsuperscript{a}, Ildar Nisamedtinov\textsuperscript{b}, Kaarel Adamberg\textsuperscript{b}, Toomas Paalme\textsuperscript{b,}\textsuperscript{*}

\textsuperscript{a}National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia
\textsuperscript{b}Tallinn Technical University, Ehitajate tee 5, 19086 Tallinn, Estonia

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Abstract

Two novel modifications of continuous culture with gradual change of dilution rate (A-stat): D-stat and auxo-accelerostat were evaluated in the studies of the effect of changing individual environmental parameters ($T$, pH, $pO_2$, substrate concentration, etc.) on growth characteristics of different microorganisms. Common for those cultivation methods is that one environmental parameter is programmed to change with constant change rate (change-stat) while the others are kept constant or in the range not affecting the growth characteristics. The environment response growth curves were obtained starting with chemostat (in A-stat and D-stat) or auxostat (in auxo-accelerostat) steady-state cultures followed by change of set-point value of the desired cultivation parameter. Physiological studies of Saccharomyces sp. and Lactococcus lactis were combined with validation of the different modifications of the A-stat method based on well-known cultivation techniques: chemostat, pH-auxostat, $pO_2$-auxostat $CO_2$-auxostat and fed-batch. The auxo-accelerostat was shown to be very efficient for cell characterization and dynamic studies in growth environments with excess of essential substrates. Choosing the rate of change of environmental parameters was shown to be critical in comparative physiological studies of microorganisms.

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1. Introduction

A common strategy in physiological studies and growth characterization has been keeping the environmental conditions as constant as possible. This has many advantages but it is often experimentally difficult or time-consuming to achieve. The alternative is to carry out the studies in well-defined changing environmental conditions. To improve the performance of chemostat experiments, the A-stat method was developed. The objective was to use smooth change of dilution rate instead of stepwise change because in the natural environments, as well as in technological processes, the growth conditions (like biomass concentration, product and substrate concentrations, etc.) change permanently. Usually, these changes are rather smooth, enabling gradual adaptation of the cells to the changing environment. Also, in batch culture, the growth-associated environmental parameters change, but the change rate is usually insignificant at low cell densities and dramatic during final stages of cultiva-
tion. The effect of the change rate on the culture characteristics is often unpredictable and difficult to study in batch culture.

The continuous culture methods, chemostat (Monod, 1950; Novick and Szilard, 1950) and turbidostat (Bryson and Szybalski, 1952) were introduced about a half century ago and have shown to be accurate methods for the determination of culture characteristics in precisely defined steady-state culture conditions. In chemostat, the dilution rate, and in turbidostat, the biomass concentration is kept constant. The auxostat cultures like pH-auxostat (Rice and Hempfling, 1985), CO$_2$-auxostat (Watson, 1969), pO$_2$-auxostat (Hospodka, 1966), etc., can be used alternatively to turbidostat for biomass control at constant level. The methods are, however, time- and medium-consuming as 4–5 culture volumes are needed to obtain the steady state (Tempest et al., 1967) and several steady-state points are required to obtain the chemostat or auxostat environment response growth curve. Nevertheless, the transition rate from one cultivation condition into another and the history of the culture can still affect the results obtained. Rieger et al. (1983) showed that in the chemostat culture of Saccharomyces cerevisiae, the stepwise change in dilution rate larger than 0.01 h$^{-1}$ near critical respiratory growth rates caused the repression of respiration. The multiplicity of stable steady states may correspond to a single operating point (Axelsson et al., 1992; Zamamiri et al., 2001). Stepwise changes of the dilution rate or environmental conditions in chemostat experiments can also cause oscillations (Fiechter and von Meyenburg, 1968) or premature washout of culture.

To keep the above-mentioned effects under control we have developed the accelerostat (A-stat) technique, where the rate of change of the dilution rate (i.e. the acceleration rate) was kept constant (Paalme and Vilu, 1992) and used this method for the physiological studies of Escherichia coli (Paalme et al., 1995). S. cerevisiae (Paalme et al., 1997a) and Lactobacillus plantarum (Kask et al., 1999). Acceleration rates of 0.005–0.02 h$^{-2}$ were used in those studies. Recently, van der Sluis et al. (2001) analysed the application of the method for estimation of the steady-state culture characteristics in the case of Zygosaccharomyces rouxii cultures and concluded that an A-stat with acceleration rate 0.001 h$^{-2}$ is attractive to be used instead of chemostat whenever a rough estimation of the steady-state culture characteristics is acceptable.

However, as mentioned above, in nature, and even in industrial processes, the steady state is rare. Therefore, the characterization of the microbial culture in gradually changing growth conditions is natural, essential and even more informative for practical purposes. The intermediate acceleration rate can serve to quickly and quantitatively compare the strains, which is increasingly important for the characterization of mutant or recombinant strains (Herwig et al., 2001). Nevertheless, using accelerostat, the rate of change should be added as an additional environmental factor to the matrix of environmental parameters for determining the values of growth characteristics of the microorganism.

In the present paper, the modifications of A-stat: D-stat and auxo-accelerostat, are introduced and the prospects of different applications for the characterization of microorganism and physiological studies are analysed. In contrast to A-stat, one of the environmental parameters (T, pH, pO$_2$, product or inhibitor concentration, etc.) was changed at constant rate. Due to the constant rate of change of the environmental conditions, the methods can be called commonly change-stat.

2. Materials and methods

2.1. Microorganisms and cultivation conditions

S. cerevisiae S288C, ALKO 734, EC1118 and C63, Saccharomyces uvarum W34, and Lactococcus lactis subsp. lactis 1387 were used in different experiments. The exact composition of mineral medium used in the experiments with S. cerevisiae and S. uvarum has been described previously (Paalme et al., 1995, 1997a). In pH-auxostat experiments with L. lactis, lactose-containing complex medium (pH = 6.8) was used (g l$^{-1}$): lactose (Reanal, Hungary)—20, tryptone (Lab M, UK)—5, yeast extract (Difco, USA)—2.5, Tween 80 (Ferak, Berlin)—1, Na-citrate—2.3, K$_2$HPO$_4$—2, MgSO$_4$·7H$_2$O—0.2, MnSO$_4$·5H$_2$O—0.04. The growth temperature in the change-stat experiments with S. cerevisiae and S. uvarum was 30°C. The specific cultivation conditions (pH, T, D, change rate) used in the different
experiments are given in the figure captions. The cell densities 0.3–0.7 g dwt l\(^{-1}\) were chosen in auxostat experiments in order to avoid the unexpected growth limitation by any growth factor and/or inhibition by products.

2.2. Cultivation system and cultivation process routines

The fermentation equipments from several companies, e.g., Applikon (Netherlands), Bioengineering

![Diagram of computer-controlled cultivation system and control algorithms used in change-stat experiments.](image)

Fig. 1. Principal scheme of computer-controlled cultivation system and control algorithms used in change-stat experiments. pmpMED1 and pmpMED2—feeding media pumps, pmpOUT—outflow pump, pmp\(_{\mathrm{pH}}\) and pmpO\(_2\)—pH control and oxygen feeding pump, \(\omega\)—stirrer, \(V\)—culture volume, \(a_D\)—acceleration rate, \(S_c\)—total amount of added substrate, \(S_0\)—initial substrate concentration in the fermenter, \(D_{\mathrm{set}}, D_{\mathrm{HIGH}}, D_{\mathrm{LOW}}, \mathrm{pH}_{\mathrm{set}}, \mathrm{pO}_2_{\mathrm{set}}, \mathrm{CO}_2_{\mathrm{set}}, \mu_{\mathrm{set}}\)—set points of dilution rate, pH, dissolved oxygen and carbon dioxide concentration, and specific growth rate, \(t\)—running time.
(Switzerland) and Braun (Germany), were used. The principal scheme of the computer-controlled cultivation system used in the change-stat experiments is shown in Fig. 1. The system was equipped with pumps, valves, balance, pH-, and \( pO_2 \), a\( CO_2 \), \( O_2 \) and T-meters with flexible cultivation control software. Software “FermExpert” (Vinter et al., 1992) and its upgrade “BioXpert” (Applikon) were used. The culture volume (150 ml–2.5 l), depending on the cultivation vessel used was kept constant using an overflow tube or level control. The samples were collected from the effluent line. For the on-line quantification of the outflow from the fermenter, the balance was used.

The environmental conditions \( Z \) (pH, \( pO_2 \), \( T \), etc., see List of symbols) were kept constant or at least in “neutral” (near optimal) range thereby not affecting the growth characteristics \( A \) (\( \mu \), \( Y_{XS} \), cell composition, etc.) by controlling cultivation conditions \( Y \) (\( T_{set} \), pH\(_{set} \), \( D_{set} \), \( \mu_{set} \), \( V_{set} \), feeding medium composition, etc.). The steady state (constant \( \mu \), \( Y_{XS} \) and cell composition, etc., in time span of the observation) was obtained by applying constant cultivation conditions (\( Y \)) for at least 4–5 culture volumes to start the change-stat. Preferably only one environmental or cultivation parameter was changed with constant change rate and the respective response growth curves were obtained.

2.3. Control algorithms

The principal control algorithms of the A-stat, D-stat, \( \mu \)-stat, and pH-, \( pO_2 \)- and \( CO_2 \)-auxo-accelerostat are given in Fig. 1. In accelerostat (A-stat), after obtaining steady state, the dilution rate (\( D \)) was increased linearly using constant change rate (acceleration rate \( a_D \)). In the case of D-stat, the dilution rate was kept constant while one of the cultivation parameters was changed with constant rate. In fed-batch (\( \mu \)-stat) experiments, immediately after the depletion of the carbon source, the control algorithm C was switched in. The set point of specific growth rate (\( \mu_{set} \)) was kept constant while one of the cultivation conditions was changed. The simple algorithms, using two-level (\( D_{LOW} \), \( D_{HIGH} \)) control of the media pump to keep the pH, \( pO_2 \) or \( CO_2 \) at the same level were applied in auxo-accelerostat experiments (algorithms D, E, F in Fig. 1). In several instances, the set-point values of \( D_{LOW} \) and \( D_{HIGH} \) were changed during the experiment either manually or automatically: for example, if condition \( D = D_{HIGH} \) was satisfied over a period of 15 min, the \( D_{HIGH} \) was increased. The set-point values of the cultivation parameters (\( D_{set} \), pH\(_{set} \), \( pO_{2set} \), \( CO_{2set} \), \( T_{set} \), etc.) were set either manually or according to the preset profile. For temperature and pH control, the ADI-1030 controller (Applikon) was used.

2.4. Analytical methods

Parallel to on-line measurements of \( pO_2 \), \( T \), composition of off-gases, pH, etc., the substrate, and product and biomass concentrations were measured off-line. Samples were taken at 0.5- to 1-h intervals. The concentrations of substrates and products in the growth medium were determined by high-pressure liquid chromatography. Samples (1 ml) were centrifuged (5000 \( \times \) g, 10 min) and the supernatants were used for analysis. Isocratic elution (0.6 ml min\(^{-1} \)) of Aminex HPX-87H (BioRad) column (300 \( \times \) 7.8 mm and a particle size of 9 \( \mu \)m) with 0.009 N H\(_2\)SO\(_4 \) was used. UV\(_{206} \) and refractive index detectors were applied in parallel enabling the measurement of the concentrations of most of the sugars and organic acids present in the culture medium. Due to the small sample size, the dry weight was calculated from optical density (OD) values using a calibration curve. Cell growth characteristics were calculated using “BioXpert” software.

3. Results and discussion

The chemostat-, auxostat- and fed-batch-based cultures were used to obtain the environment response growth curves. The constant rate of change of the desired cultivation parameter was used to study and compare the applicability of different change-stat methods for characterization of microbial cultures.

3.1. Chemostat-based cultures with programmed change rate

The effect of each individual environmental parameter on growth was studied using two principally different experimental setups: (i) changing the concentration of the growth-limiting substrate by chang-
ing the dilution rate and keeping other environmental parameters constant (A-stat); (ii) keeping the dilution rate constant and changing one of the environmental parameters (D-stat).

3.1.1. Accelerostat (A-stat)

The environmental conditions in accelerostat are determined by dilution rate, rate of change of dilution rate, composition of the feeding medium and set-point values of $T$, pH, $pO_2$, $V$, stirrer speed and aeration rate, which were all kept constant except dilution rate. In response to $dD/dt = a_D$ (change of dilution rate or acceleration rate), the environmental parameters substrate concentration ($S_i$) and product concentration ($P_i$) changed. It can be predicted, according to the Monod equation (Monod, 1949) and simulated behaviour of substrate concentration in accelerostat culture (van der Sluis et al., 2001) that the concentration of the limiting substrate increases at moderate acceleration rates $(0.005–0.02 \text{ h}^{-1})$ almost linearly, at least up to critical growth rates.

We used A-stat mode to study the transitory metabolism (from respiratory to respiro-fermentative and to fermentative) of $S.\ cer\v v\ i\ s$. For example, in the accelerostat culture of $S.\ cer\v v\ i\ s$ S288C ($a_D = 0.022 \text{ h}^{-1}$), the accumulation of acetate started a short time after the $pO_2$ breakpoint, indicating the exhaustion of respiratory capacity; the ethanol accumulation started after about 1.5 h and consumption of acetate about 3 h later (Fig. 2A). In accelerostat cultures of $S.\ cer\v v\ i\ s$ ALKO743 ($a_D = 0.015 \text{ h}^{-1}$), the ethanol accumulation started immediately after the exhaustion of respiratory capacity (Fig. 2B). Similar behaviour was reported in accelerostat experiments of $S.\ cer\v v\ i\ s$ CEN.PK 113-7D ($a_D = 0.0075 \text{ h}^{-1}$), however, in experiments with corresponding strain, the accumulation of ethanol and acetate was simultaneous if the rate of change of dilution rate was stepwise (Herwig et al., 2001). Thus, the on-set of acetate and ethanol accumulation can depend on both the change rate and the strain of bacteria.

In A-stat experiments the minimum concentration of glucose decreasing the respiratory capacity can be determined at the onset of $Q_{O_2}$ decrease (Fig. 2B) and that of complete catabolic repression at the point of onset of glycerol accumulation (Fig. 2C). The ethanol concentration at $Q_{O_2}$ breakpoint (Fig. 2B) is a technologically very important parameter, as it determines the maximum safe set-point value of ethanol concentration for control loops used in fed-batch production processes of baker’s yeast. Results show that accelerostat is a suitable method to study technologically very important transitory features of the metabolism of different yeast strains.

The acceleration rate is very important as, with stepwise change, too high acceleration rates can cause the accumulation of inhibitory products even at specific growth rates lower than critical growth rate ($\mu_{\text{crit}}$). On the other hand, using a too slow rate of change the dynamic cellular processes, important for technological characterization of the strains, may be masked. Results of our experiments using different microbial cultures (Paalme et al., 1997a,b; Kask et al., 1999) and also cell lines (Drews et al., 1997) suggested that the effect of acceleration rate depends on maximum specific growth rate ($\mu_{\text{max}}$). For most practical purposes (for determination of critical and maximum growth rate and growth yields), acceleration rates remaining in the range of $0.01–0.04 \mu_{\text{max}}$ were suitable.

3.1.2. D-stat

In contrast to accelerostat, the dilution rate in D-stat was kept constant and one of the environmental parameters was changed at constant rate. Growth of $S.\ uvar\ u m$ W34 in D-stat with a gradual increase of growth temperature from 30 to 50 $^\circ\text{C}$ ($a_T = 2 \text{ \degree\text{C}\ h}^{-1}$) at constant dilution rate ($D = 0.07 \text{ h}^{-1}$) is shown in Fig. 3A. The constant gradual increase of temperature resulted in a decrease in the biomass concentration and growth yield ($Y_{XS}$) due to the accumulation of the acetate and ethanol, and an increase in substrate concentration ($S_i$). The decrease of specific growth rate ($\mu$) at constant dilution rate ($D$) can explained by the formula:

$$\mu = D + \frac{dX}{dY} = D + \frac{d(Y_{XS}(S_f - S_i))}{dY_{XS}(S_f - S_i)} \quad (1)$$

In the D-stat experiment with $S.\ cer\v v\ i\ s$ EC1118 with a decrease in the nitrogen to carbon ratio (N/C) in the feed medium at a change rate of $a_{N/C} = -0.01 \text{ mol}\text{mol}^{-1}\text{h}^{-1}$, the accumulation of acetate and ethanol and decrease of growth rate started simultaneously after N/C had reached the value of 0.04 (Fig. 3B). Decrease of nitrogen supply caused the transition
Fig. 2. A-stat experiments (mineral medium with glucose 10 g l\(^{-1}\), pH\(_{st}\) = 4.6) with (A) *S. cerevisiae* S288C (\(a_D = 0.022\) h\(^{-1}\)), (B) *S. cerevisiae* ALKO 734 (\(a_D = 0.015\) h\(^{-1}\)) and (C) *S. uvarum* W34 (\(a_D = 0.015\) h\(^{-1}\)). \(D\)—dilution rate (h\(^{-1}\)), \(\mu\)—specific growth rate (h\(^{-1}\)), \(\mu_{\text{crit}}\)—critical growth rate (h\(^{-1}\)), \(pO_2\)—dissolved oxygen concentration (%), \(X\)—biomass concentration (g dwt l\(^{-1}\)), eth—ethanol concentration (g l\(^{-1}\)), glc—glucose concentration (g l\(^{-1}\)), glr—glycerol concentration (g l\(^{-1}\)), \(Q_{\text{ace}}\)—specific acetic acid production rate (mmol g dwt\(^{-1}\) h\(^{-1}\)), \(Q_{O_2}\)—specific oxygen consumption rate (mmol g dwt\(^{-1}\) h\(^{-1}\)), CR—onset of catabolic repression.
from the C limitation into N limitation and a concomitant decrease of $S_f/C_0$ and specific growth rate due to the insufficient supply of nitrogen.

The experiments carried out using D-stat with a gradual programmed change in cultivation parameters showed good potential of the method in determination of critical values of environmental (for example, temperature) or cultivation factors (for example, relative nitrogen, vitamin, microelement, etc., concentration in the feed), which cause a decrease in specific growth rate down to the dilution rate. This method is effective for detection of the limits of environmental conditions and for balancing the culture medium.

The experiments clearly showed that the both A-stat and D-stat suited well for studies of the growth characteristics. However, in environments with excess of essential substrates, neither D-stat nor A-stat was satisfactory.
3.2. Auxostat-based cultures: pH-, pO2- and CO2-auxo-accelerostat

The behaviour of growth characteristics in changing environments with surplus essential growth substrates was studied in auxostat cultures using pH-, pO2- and CO2-auxo-accelerostat modes. The steady-state cultures were obtained using the algorithms D, E or F (Fig. 1) and passing 5–10 volumes of the culture medium through the fermenter until constant dilution rate and biomass concentration were obtained. The biomass concentration was kept below the inhibitory level by adjusting appropriate biomass-related set-point values (pHset, pO2set or CO2set). In the case of pH-auxostat, the growth yield and buffering capacity of the cultivation medium mainly affected the biomass proportionality coefficient. In the case of pO2- and CO2-auxostat, the proportionality coefficient was dependent also on the specific growth rate.

3.2.1. pH-auxo-accelerostat

pH-auxo-accelerostat was technically the simplest, most reliable and convenient setup for most applications. Experiments being carried out in batch and chemostat cultures have shown the validity of the specific rate of proton production as a formal control parameter (Vicente et al., 1998). The biomass concentration in pH-auxostat cultures was determined from the amount of alkali (nALK) required for the titration of the pH of the culture broth back to its initial pH value and from the biomass yield based on acid formation YAC (X=nALKYAC). We used the method to study the effect of pH, T, pO2, water activity, inhibitory substances (Ii) and tryptone or yeast extract on the growth of S. cerevisiae S288C and L. lactis subsp. lactis 1387. Using pH-auxostat, the change of environmental parameters had only a relatively small effect on biomass concentration at near optimal growth conditions. At extreme conditions, however, due to the change of YATP the effect was significant. For example, in pH-auxostat culture of L. lactis subsp. lactis 1387, with a gradual increase in temperature from 25 to 40 °C after reaching the temperature optimum (38 °C), the YATP (YLAC) and biomass concentration started to decrease (Fig. 4). However, a change in biomass concentration in auxo-accelerostat was not wanted because it affects negatively the precision of calculation of growth characteristics (in case of constant biomass concentration, the component dYATP/dt in Eq. (1) equals to 0).

Only in specific cases, for example, in studies of the effect of compounds having high buffering capacity, for example, salts of “weak acids” like formate, acetate, etc., the change in biomass concentration can seriously complicate the measurements of growth characteristics. In this case, and in cases when the growth

![Graph](image-url)
has no significant effect on the pH of the media, the other auxostat methods such as \( pO_2 \)-auxostat or \( CO_2 \)-auxostat can be used instead.

### 3.2.2. \( pO_2 \)-auxostat

\( pO_2 \)-auxostat, like pH-auxostat, was precise, sensitive and convenient to use in experiments with a programmed change of environmental conditions. Under constant cultivation conditions, the volumetric oxygen consumption \( q_{O_2} = X\mu / Y_{XO} \) is equal to oxygen transfer rate \( r_{O_2} = \kappa (100 - pO_2) \), so biomass concentration is equal to:

\[
X = \kappa (100 - pO_2) Y_{XO} / \mu \tag{2}
\]

where \( \kappa \) is the oxygen transfer coefficient and \( Y_{XO} \) is the biomass yield based on oxygen consumption. The biomass concentration is proportional to the \( \Delta pO_2 \) under constant \( \mu \), \( Y_{XO} \) and temperature. In the experiments with a gradual change of environmental factors, the growth yield and oxygen transfer coefficient may vary significantly and thus lead to a change in biomass concentration. For example, increasing the concentration of tryptone in the feed medium of \( pO_2 \)-auxo-accelerostat culture of \( S. cerevisiae \) S288C increased the biomass concentration in parallel to the growth yield (Fig. 5). The increase of biomass concentration can be explained, according to Eq. (2), by the interplay of \( Y_{XO} \) and \( \kappa \) with increase of tryptone concentration as well as solubility of oxygen. The increase of tryptone in the feed can increase the growth yield based on oxygen consumption (\( Y_{XO} \)), as the tryptone would be providing some components that cells do not need to synthesize.

### 3.2.3. \( CO_2 \)-auxostat

As an alternative to pH- or \( pO_2 \)-auxostat, the \( CO_2 \)-auxostat was used in studies of fermentative growth of yeast. \( CO_2 \) concentration in the exhaust gas, which metabolically is related to the energy production rate, was proportional to the biomass concentration at near maximum growth rates. Compared with the \( pO_2 \)-auxostat, and especially with the pH-auxostat, the \( CO_2 \)-auxostat had significant delay in measurements of \( CO_2 \) production rate, which may become critical when applying high rates of change. Nevertheless, the method was suitable for most applications. For example, it was used successfully to study the effect of monocarboxylic acids, maintaining constant \( pO_2 \) and \( pH \) by means of regulating stirrer speed and adding titrant.

The experiments using pH-, \( CO_2 \)- or \( pO_2 \)-auxo-accelerostat showed that all the methods might be successfully used for determination of the effects of environmental conditions on the growth character-

![Fig. 5. \( pO_2 \)-auxo-accelerostat cultivation of \( S. cerevisiae \) S288C (mineral medium with glucose 50 g l\(^{-1}\), \( pH_{set} = 3.6 \)) with smooth increase of tryptone concentration (\( c_{tryp} = 0.23 \) g l\(^{-1}\)). \( X \)—biomass concentration (g dwt l\(^{-1}\)), \( \mu \)—specific growth rate (h\(^{-1}\)), \( Y_{ATP} \)—growth yield based on ATP production (g dwt l\(^{-1}\) mol-ATP l\(^{-1}\)), \( pO_2 \)—dissolved oxygen concentration (%).](image-url)
istics. When selecting the method and experimental conditions (media buffering capacity, biomass-related set-point value of pH, \( pO_2 \) or \( CO_2 \), studied range of environmental conditions, etc.), the possible change of biomass concentration should be minimized, as the change of biomass concentration decreases the preciseness of the calculations of the growth characteristics. Also, the effect of biomass concentration itself on the growth environment should be considered. The problem is best solved using a sensitive and stable detector for direct biomass measurement thereby keeping the biomass concentration constant, as in the case of the turbidostat.

3.3. Fed-batch-based modifications of A-stat

The A-stat approach was also used with fed-batch cultures. Two different strategies were employed: (i) increasing the culture volume exponentially by feeding with initial cultivation medium (Fig. 1, algorithm B for fed-batch) and (ii) increasing exponentially the substrate feed rate by feeding with highly concentrated growth-limiting substrate (Fig. 1, algorithm C).

The feeding with initial cultivation medium (i) can be used to reduce the amount of inoculum, culture medium and time to obtain the steady-state culture, as our previous experiments with insect cell culture have shown. The quasi-steady-state culture (Kalogerakis and Boyle, 1981) was obtained accordingly to a set-point value of feeding rate in D-stat (Drews et al., 1997) or in CO2-auxo-accelerostat mode keeping the volumetric CO2 production rate constant (Drews et al., 1998).

Feeding with high substrate feeding medium (ii), an exponential increase of biomass concentration at specific growth rate (\( \mu \)) equal to the specific substrate feeding rate can be obtained (Paalme et al., 1990). The critical respiratory growth rate (\( \mu_{crit} \)) of industrial baker’s yeast \( S. \) cerevisiae C63 was determined by changing the set-point value of the specific growth rate (\( \mu_{set} \)) at a constant rate (0.0109 h\(^{-2}\)) in fed-batch (\( \mu \)-stat) (Fig. 6). As in A-stat cultures of \( S. \) cerevisiae ALKO 734 (Fig. 2B), the accumulation of ethanol occurred at the critical growth rate.

In general, the results demonstrated that, the fed-batch techniques using programmed change of culture environment are effective in industry-orientated optimisation and control processes, for accurate physiological studies and cell characterization the continuous culture methods had overwhelming advantages.

3.4. Effect of change rate of environmental conditions on growth characteristics

As already mentioned, the high change rates of cultivation or environmental parameters can signifi-
cantly affect the growth curves and accuracy of measurement in change-stat experiments with gradual change of desired environmental parameter. The effect of rate of change on the growth curve is well illustrated by studying the effects of “weak organic acids” on the growth of *S. cerevisiae* S288C in the auxo-accelerostat (Fig. 7). The mode of action of monocarboxylic acids and the stress response of yeast cells depended on the amount and feeding rate of these compounds. For example, if the concentration of formic acid was increased with a rate of change of 0.69 g l$^{-1}$ h$^{-1}$, the specific ethanol production rate ($Q_{\text{eth}}$) decreased, probably due to a decrease of internal pH. However, in the case of a slower increase of formic acid (0.25 g l$^{-1}$ h$^{-1}$), the $Q_{\text{eth}}$ increased. The yeast cells manage to maintain homeostasis by inducing membrane transporters to pump out the H$^+$ and anions and/or decrease the permeability of membranes to the weak organic acids (Piper et al., 2001). This clearly shows that the cells require some time to adapt to the environmental conditions, and thus, by using different rates of change, different cell responses can be obtained.

The classical chemostat and auxostat techniques eliminate the rate of change as an additional parameter by combining the environmental response curves from steady-state or exponential growth points. In change-stat experiments, choosing very low rates of change can also eliminate the effect of rate of change. However, the question always remains: is the rate of change sufficiently low to maintain the steady state and eliminate the effect of rate of change itself? The classical criterion for the steady-state, constant biomass concentration, was shown to be unsuitable in change-stat experiments. Constant growth yields ($Y_{\text{ATP}}, Y_{\text{XS}},$ etc.) are more suitable. However, they are also not sufficient. To exclude the effect of rate of change on response curves in change-stat experiments, additional change-stat experiment(s) with different change rate(s) should be carried out. Only, if the same environment response curves $A=f(Z)$ will be obtained at two different change rates of cultivation parameter, for example, at change rate $a_i$ and $0.5a_i$ can we be certain that the change rate itself had no effect.

On the other hand, the change rate of environmental factors such as $T/dt$ or pH/$dt$ or $pO_2/dt$, etc., is also an essential environmental parameter, and the only principal advantage of using change rate near to 0 is that it makes the results of different experiments more easily comparable. For comparative experiments, the change rate should be related to maximum specific growth rate at current growth conditions. Our experience based on over a hundred different change-stat experiments has shown that for primary and comparative characterization of microorganisms, the use of the following change rates, $2 \text{ } ^\circ\text{C} \mu_{\text{max}}$ for
The preciseness of measurements is the critical aspect complicating the application of the accelerostat, D-stat and auxo-accelerostat compared with steady-state chemostat cultures especially at high change rates. In this respect, the auxo-accelerostat is more complicated than D-stat and accelerostat, as dilution rate behaves according to the unknown function, while in accelerostat the behaviour of dilution rate can be described by linear equation. In addition, the precise control of the culture volume and culture parameters (OD, $pO_2$, pH, etc.) is crucial.

The experiments clearly showed that the A-stat works best in environmental conditions, where the increase of limiting substrate concentration as well as growth yield remains in the linear range and product concentrations do not reach the inhibitory level. The surplus of substrates can be reached at $D > \mu$ in washout conditions, but in this stage, the biomass, substrate and product concentrations change dramatically and therefore the accuracy of measurements of growth characteristics decreases. In turn, the auxo-accelerostat, which is not suitable for studies in substrate-limiting conditions, is very convenient for studies in surplus of essential substrates. The performance and application range of change-stat methods is summarized in Table 1.

### Table 1: Performance of accelerostat (A-stat), D-stat and auxo-accelerostat

<table>
<thead>
<tr>
<th>Application</th>
<th>A-stat</th>
<th>D-stat</th>
<th>Auxo-accelerostat</th>
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<tr>
<td>Chemostat curves</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Determination of critical growth rate</td>
<td>+++</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Determination of maximum growth rate</td>
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<td>+++</td>
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<tr>
<td>Determination of maintenance energy</td>
<td>+</td>
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<tr>
<td>Dynamic growth characterization</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Effect of nonessential substrates</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Effect of environmental parameters on growth yield</td>
<td>±</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Effect of environmental parameters on growth rate</td>
<td>±</td>
<td>±</td>
<td>+++</td>
</tr>
<tr>
<td>Balancing the cultivation media</td>
<td>±</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Applicability in fed-batch mode</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

(-) Ineffective in practice, (±) possible under certain circumstances, (+) satisfactory, (++) good, (+++) very good.

### 4. Conclusions

The A-stat, D-stat and auxo-accelerostat (change-stat methods) are all very effective for characterization of cell lines and microbial strains under dynamic growth conditions as well as for overall physiological studies. The rate of change of environment is an important environmental parameter affecting the culture characteristics. Both chemostat- and auxostat-based methods developed can be used to study the effect of change rate. Still, a lot of work both technical and theoretical should still be done to make change-stat a common routine in industrial and scientific practice.

### 5. List of symbols

**Growth characteristics (A)**

- $\mu$ specific growth rate $(h^{-1})$
- $\mu_{crit}$ specific growth rate of transition from one physiological state into another $(h^{-1})$
- $\mu_{max}$ maximum specific growth rate $(h^{-1})$
- $Q_{O_2}$ specific oxygen consumption rate $(\text{mmol g dwt}^{-1} h^{-1})$
- $Q_{CO_2}$ specific CO$_2$ production rate $(\text{mmol g dwt}^{-1} h^{-1})$
- $Q_{eth}$ specific ethanol production rate $(\text{mmol g dwt}^{-1} h^{-1})$
- $Q_{ace}$ specific acetic acid production rate $(\text{mmol g dwt}^{-1} h^{-1})$
- $Q_{lac}$ specific lactic acid production rate $(\text{mmol g dwt}^{-1} h^{-1})$
- $Y_{XS}$ biomass yield based on substrate consumption $(\text{g dwt mol}^{-1})$
- $Y_{ATP}$ biomass yield based on ATP production $(\text{g dwt mol}^{-1})$
- $Y_{LAC}$ biomass yield based on lactic acid production $(\text{g dwt mol}^{-1})$
- $Y_{AC}$ biomass yield based on the acid production $(\text{g dwt mol}^{-1})$
- $Y_{OXO}$ biomass yield based on oxygen consumption rate $(\text{g dwt mol}^{-1})$

**IC$_{50}$ inhibitory concentration 50% $(l^{-1})$**
Environmental parameters (Z)

- \( a_i \): change rate of any environmental parameter (\( dT/dt, dS_i/dt, dP_i/dt \), etc.)
- \( pH \): \( pH \) of the culture medium
- \( T \): temperature (°C)
- \( OD \): optical density at 540 nm
- \( X \): biomass concentration in the culture medium (g dry wt l\(^{-1}\))
- \( pO_2 \): dissolved oxygen concentration (percentage of air saturation)
- \( CO_2 \): carbon dioxide concentration (%)
- \( S_i \): concentration of substrate \( i \) in the culture medium (g l\(^{-1}\))
- \( P_i \): concentration of product \( i \) in the culture medium (g l\(^{-1}\))
- \( I_i \): concentration of the growth inhibitor \( i \) in the culture medium (g l\(^{-1}\))

Cultivation parameters (Y)

- \( V \): culture volume (l)
- \( a_D \): change rate of dilution rate (acceleration rate) (h\(^{-2}\))
- \( a_{N/C} \): change rate of the N/C ratio in the feeding (mol mol\(^{-1}\) h\(^{-1}\))
- \( D \): dilution rate (h\(^{-1}\))
- \( D_{LOW} \): set point of the low value of dilution rate (h\(^{-1}\))
- \( D_{HIGH} \): set point of the high value of dilution rate (h\(^{-1}\))
- \( D_{set} \): set point of dilution rate (h\(^{-1}\))
- \( T_{set} \): set point of temperature (°C)
- \( V_{set} \): set point of culture volume (l)
- \( pH_{set} \): set point of pH
- \( pO_{2set} \): set point of dissolved oxygen (%)
- \( CO_{2set} \): set point of carbon dioxide (%)
- \( \mu_{set} \): set point of specific growth rate (h\(^{-1}\))
- \( S_f \): substrate concentration in the feeding medium (g l\(^{-1}\))
- \( S_0 \): initial substrate concentration in the fermentor (g l\(^{-1}\))
- \( S_c \): total amount of added substrate (g l\(^{-1}\))
- \( t \): running time (h)
- \( k \): biomass proportionality coefficient (g dry wt U\(^{-1}\))
- \( \kappa \): oxygen transfer coefficient (mmol l\(^{-1}\) h\(^{-1}\))
- \( q_{O_2} \): volumetric oxygen consumption rate (mol l\(^{-1}\) h\(^{-1}\))
- \( r_{O_2} \): oxygen transfer rate (mmol l\(^{-1}\) h\(^{-1}\))

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References


