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Effect of changing environmental conditions on the fermentative growth of *Saccharomyces cerevisiae* S288C: Auxo-accelerostat study

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Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any degree or examination.

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ABSTRACT

The technological processes of ethanol, wine, beer and bread production all rely on fermentative growth of *Saccharomyces cerevisiae*. The effect of environmental conditions on the fermentative growth of yeasts, essential for optimisation of the process, has been studied mostly in batch and/or anaerobic chemostat cultures.

In this study a novel auxostat based cultivation technique, auxo-accelerostat, was developed and used to map the effect of smoothly changing environmental conditions on the fermentative growth and metabolic activity of *S. cerevisiae* S288C. Various environmental factors (pH, pO_2, temperature, salinity, concentration of ethanol, propanol, tryptone, yeast extract and aliphatic monocarboxylic acids) were studied.

The effect of the rate of change of the environmental parameters on the growth of yeasts was clearly demonstrated in auxo-accelerostat experiments with increasing the concentration of monocarboxylic acids: it was shown that the toxic effect depends not only on the nature of the monocarboxylic acid but also on the rate of addition of this acid into the culture medium.

A slow decrease in the culture pH and dissolved oxygen concentration below the optimum values or increase in monocarboxylic acid concentration in the culture medium caused a decrease in growth rate mainly due to the increase in the energy costs for maintaining homeostasis and not by the inhibition of the energy producing reactions. A slow increase in the concentration of biomass, propanol and NaCl as well as a fast increase in monocarboxylic acids lead to a decrease in glycolytic activity (Q_{ATP}).

Auxo-accelerostat experiments showed that in optimal conditions in mineral medium with excess of glucose (introducing fermentative mode of growth), the capacity of ATP production was not obviously a growth rate-limiting step for *S. cerevisiae* and glycolytic activity was controlled by the demand for ATP. This assumption was supported by the fact that slow addition of monocarboxylic acids to the culture medium was accompanied by up to 80% increase in the specific ATP production rate, i.e. the cells were able to increase the energy production as a response to a rise in maintenance costs. Moreover, as the slow addition of tryptone or yeast extract into the culture medium (providing the precursors) increased the specific growth rate, it could be assumed that the growth rate limiting step for yeast cells at fermentative growth is the synthesis of monomers (e.g., amino acids, nucleotides) and their polymerisation.

The present study demonstrates that the auxo-accelerostat cultivation method, besides being an efficient tool for the quantification of culture characteristics, is also a promising method allowing elucidation of the growth rate control mechanism(s) in cells.

Keywords: *Saccharomyces cerevisiae* S288C, fermentative growth, quantitative growth characteristics (μ, Q_{ATP}, Y_{ATP}), auxo-accelerostat, changing environmental conditions, rate of change, stress response, adaptation

LIST OF PAPERS

This Thesis is based on the following papers referred to by their Roman numerals in the text. Some unpublished data are also presented.

I Drews, M., Kasemets, K., Nisamedtinov, I., Paalme, T. 1998. Continuous cultivation of insect and yeast cells at maximum specific growth rate. *Proceedings of Estonian Academy of Sciences, Chemistry* 47, 175–188.

II Kasemets, K., Drews, M., Nisamedtinov, I., Adamberg, K., Paalme, T. 2003. Modification of A-stat for the characterization of microorganisms. *Journal of Microbiological Methods* 55, 187–200.

III Kasemets, K., Kahru, A., Laht, T.-M. and Paalme, T. Study of the toxic effect of the short- and medium-chain monocarboxylic acids on the growth of *Saccharomyces cerevisiae* using CO_2-auxo-accelerostat fermentation system. *International Journal of Food Microbiology, submitted*

IV Kasemets, K., Nisamedtinov, I., Laht, T. -M., Abner, K. and Paalme, T. Behaviour of growth characteristics of *Saccharomyces cerevisiae* S288C in changing environmental conditions: auxo-accelerostat study. *Antonie van Leeuwenhoek, submitted*

V Adamberg, K., Kasemets, K. and Paalme, T. 2003. Auxo-accelerostat – a new effective cultivation system for culture characterization. *In*: S. Sorvari (Ed.), Proceedings of the 1st International Congress on Bioreactor Technology On Cell, Tissue Culture and Biomedical Application. Karhukopio OY, Turku, Finland, p. 115–125.

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INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is an important biotechnological organism e.g. for the production of ethanol, recombinant proteins, antibiotics or biomass. For thousands of years, *S. cerevisiae* has played a central role in food production and conservation thanks to its ability to ferment glucose to ethanol and carbon dioxide. In pharmacy and chemistry, a large number of substances (enzymes, polysaccharides, growth factors, etc.) are extracted from this organism. In addition, due to genetic engineering, manipulated yeasts now produce some medicines. For example, vaccines against hepatitis B surface antigen are produced by *S. cerevisiae* using recombinant technology.

Apart from its industrial value, yeast is a very useful model for scientists, as a simple, unicellular eukaryote developed to a unique powerful model system for biological research. Its prominent useful features are cheap and easy cultivation, short generation times, detailed genetic and biochemical knowledge accumulated in many years of research. An international consortium completed the sequencing of the *S. cerevisiae* strain S288C genome in 1996. It was the first eukaryotic genome that was completely sequenced.

During the last decades more attention has been paid to the physiology and metabolic function and regulation in this organism. The studies of the links between macroscopic bioenergetic parameters and cell physiology show how the microorganisms grow, adapt or die, while the physiological and molecular mechanisms responsible for these processes constitute an emerging field of research.

The current thesis focuses on the effect of smooth and constant change in environmental conditions (pH, pO_2, temperature, salinity, concentration of biomass, ethanol, propanol, tryptone, yeast extract and aliphatic monocarboxylic acids) on the fermentative growth and metabolic rates of *S. cerevisiae* S288C. A novel continuous cultivation method, auxo-accelerostat, was applied in these studies. An overview is given of published data on the physiological and biotechnological aspects of carbon and energy metabolism, general effect of environmental conditions on the growth of *S. cerevisiae* and the main cultivation strategies in physiological studies. The results and discussion part summarizes the yeast cell responses to continuously changing environmental conditions and growth strategies in substrate excess conditions. The novel continuous cultivation technique, auxo-accelerostat, is described.

LITERATURE REVIEW

1.1. *Saccharomyces cerevisiae* – general aspects of physiology

Saccharomyces cerevisiae is one of the simplest unicellular eukaryotic organisms. Subcellular compartmentalization in *S. cerevisiae* typifies that of eukaryotic cells with the following structures present: nucleus, mitochondria, Golgi apparatus, secretary vesicles, endoplasmatic reticulum, and vacuoles. Although some of the features are not characteristic for higher eukaryotic cells as the budding, which is a unique mode of reproduction and thick cell wall.

The yeast cell envelope surrounds the cytoplasm and consists of plasma membrane, periplasmic space and, the cell wall. The cell wall (main constituents are polysaccharides glucans, mannans and minor proportion of chitin) maintains the structure and the rigidity of the cells, but is freely permeable for solutes smaller than 600 Da (Scherrer et al., 1974). The periplasmic space mainly comprises the secreted proteins, including the glucoprotein enzymes invertase and acid phosphatase.

The plasma membrane forms a relative impermeable barrier for hydrophilic molecules, although is permeable for lipophilic compounds. The lipid composition of the plasma membrane is complex (mainly phospholipids and sterols) and tightly regulated, suggesting that lipids play a role in the activity of the proteins in the plasma membrane (Rest et al., 1995). *S. cerevisiae* can exhibit variations in the membrane lipid composition; particularly the unsaturated fatty acid constitution can change dramatically with changing growth conditions (Rest et al., 1995).

The primary functions of yeast plasma membranes are to dictate what enters and what leaves the cytoplasm. Specialized proteins mediate the selective uptake and/or secretion of solutes (transport of sugars, amino acids, ion translocation, etc.) across this membrane (Grenson, 1992; Lagunas, 1993). In *Saccharomyces cerevisiae*, the H^+-ATPase is the most abundant plasma membranes protein, constituting over 20% of total membrane proteins (Holyoak et al., 1996). The proton translocating ATPase in the plasma membrane of yeast creates the transmembrane electrochemical proton gradient, which is a driving force in the uptake of nutrient and also involved in the regulation of intercellular pH by pumping protons out of the cells (Serrano, 1986; Holyoak et al., 1996; Piper et al., 2001). Several stressors such as ethanol (Rosa and Sá-Correia, 1991), supraoptimal temperature (Carmelo et al., 1996), weak organic acids (Stratford and Anslow, 1996; Piper et al., 2001) stimulate *in vivo*, the activity of this membrane enzyme.

Yeast cells contain mitochondria, which structurally resemble those organelles found in higher eukaryote. Yeast mitochondria are dynamic organelles, whose size, shape and number change according to the growth conditions (Visser et al., 1995). Under aerobic conditions, yeast mitochondria are primarily involved in ATP synthesis during respiration. Although mitochondria are assumed to be energetically non-functional in the anaerobic conditions or fermentative growth, still the mitochondria have the essential role in synthesis of membrane lipids and ergosterol and biosynthesis of amino acids in these conditions (O'Connor-Cox et al., 1996).

Yeasts have simple nutritional needs. *S. cerevisiae* has a relatively narrow range of sugars, which can be considered as good growth and fermentation substrates – glucose, fructose, mannose, galactose, sucrose and maltose. Other carbon substrates (e.g. ethanol, glycerol, acetate) can act as respiratory substrates only. In addition, they also require a nitrogen source such as ammonium sulphate and minerals in form of simple salts to build up the biomass. A variety of nitrogen compounds – amino acids, peptides, purines, pyrimidines and amines can also provide the nitrogen requirements of the yeast cells (Walker, 1997). The other complex compounds that they require for growth are the vitamins (biotin, thiamine, pantothenic and nicotinic acid).

1.2. Carbon and energy metabolism in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a chemo-organotrophic microorganism, which derives their chemical energy, in the form of ATP, from breakdown of organic compounds and employs sugars as their preferred carbon and energy sources. Catabolic reactions in yeast (glycolysis, citric acid cycle, pentose phosphate cycle, etc.) provide yeast with energy, together with precursor molecules and reducing power for biosynthetic pathways.

Control of carbohydrate metabolism in *S. cerevisiae* is of both fundamental and practical significance and has been the subject of several reviews over the past decades (Käppeli and Sonnleitner, 1986; van Dijken and Scheffers, 1986; Alexander and Jeffries, 1990; Entian and Barnett, 1992; Fiechter and Seghezzi, 1992; Gancedo, 1992; Pronk et al., 1996; Hoek et al., 2000). In S. *cerevisiae* the carbohydrate metabolism depends on the availability of glucose and oxygen, and these factors are linked to the expression of several regulatory phenomena (e.g. Pasteur and Crabtree effect). Pasteur effect relates oxygen with kinetics of yeast sugar catabolism and was originally defined by Pasteur (1861) as: "The reduction of the fermentation of yeast when atmospheric oxygen is admitted in favour of the cell respiration setting in" (Walker, 1997). However, this phenomenon is only observable when glucose concentration are low or under certain nutrient-limited conditions (Lagunas, 1979).

In *S. cerevisiae*, high sugar concentrations trigger alcoholic fermentation, even under fully aerobic conditions (De Deken, 1966; Petrik et al., 1983; Hoek et al., 1998). This phenomenon is known as Crabtree effect or glucose effect and named after Herbert Crabtree, who noted in 1929 “the glycolytic activity of tumour cells exerts a checking effect on their respiration” (Alexander and Jeffries, 1990). It has been shown that Crabtree effect in *S. cerevisiae* i.e. ethanol formation in the presence of oxygen can be explained by the limited capacity of respiratory resulting in overflow of pyruvate into ethanol formation reactions and/or by repression of respiration (De Deken, 1966; Rieger et al., 1983; Käppeli et al., 1985; Sonnleitner and Käppeli, 1986; Alexander and Jeffries, 1990). Therefore the Crabtree-effect in yeasts denotes the regulatory phenomena leading to aerobic ethanol formation: glucose-sensitive yeast like *S. cerevisiae* show aerobic formation of ethanol in the presence of surplus glucose (Fiechter et al., 1981); glucose insensitive yeast like *Trichosporon cutaneum* degrade glucose exclusively in a respirative manner (Käppeli and Fiechter,

1982) and the intermediate type like *Candida tropicalis* produces ethanol only under oxygen limitation (Fiechter et al., 1981).

When the respiratory capacity is saturated, ethanol is formed and ATP produced simultaneously by fermentation and by respiration, therefore the corresponding physiological state was referred to as oxidoreductive or respiro-fermentative glucose metabolism (Käppeli et al., 1985). This hyper-flow reaction can observed as the first and fast response (short-term adaptation), which occurs after addition of glucose to a respiring culture and after a step increase in dilution rate during a continuous cultivation. The long-term cultivation of *S. cerevisiae* at high dilution rates and glucose concentrations induces the repression of the synthesis of various respiratory, TCA and gluconeogenetic enzymes and/or inactivating respiratory enzymes and sugar transport (Fiechter et al., 1981; Fiechter and Seghezzi, 1992; Entian and Barnett, 1992; Lagunas, 1993; Wills, 1996; Postma et al., 1989; Cortassa and Aon, 1998). The nature of the signal(s) causing the repression of respiration in question is at present unclear and is studied intensively.

Respirative growth

A completely respiratory sugar metabolism of *S. cerevisiae* is only possible in aerobic cultures grown under sugar limitation at relatively low specific growth rates (Petrik et al, 1983). The sugar breakdown is oxidative through the glycolysis, tricarboxylic acids cycle and respiratory chain. Final product of glycolysis pathway is pyruvate and total energy yield is 2 mole of ATP and 2 molecules of NADH per mole of glucose. During respiration, pyruvate is oxidatively decarboxylated by pyruvate dehydrogenase complex (PDH, EC 1.2.4.1) in mitochondria and coupled to coenzyme A, thus entering the tricarboxylic acid cycle and undergoing endoxidation. Most ATP generated aerobically comes from the oxidation of NADH and $FADH_2$ in the electron transport chain by oxidative phosphorylation. Calculation of the energy requirements during aerobic growth is complicated because an estimation of the P/O ratio (the value showing the number of ATP molecules produced per oxygen reduced) is required. In the calculation of total ATP production, the value of 2 for the P/O ratio has suggested for *S. cerevisiae* mitochondria, assuming the lack of the phosphorylation site corresponding to site I, the first of the three energy coupling sites in the animal respiratory chain (Oura, 1973). Actually, during respiratory growth of *S. cerevisiae* the P/O ratio is not exactly known and can change with environmental conditions and as a result the P/O values of 1–3 have been reported (Oura, 1973; Alexander and Jeffries, 1990; Fitton et al., 1994; Paalme et al., 1997b; Dejean et al., 2002).

It is possible to obtain fully respiratory growth of *S. cerevisiae*, by employing sugar-limited feed-controlled cultivation methods, such as sugar limited continuous or fed-batch cultivations (Fiechter et al., 1981; Käppeli, 1986; Pronk et al., 1996), as long as the specific sugar consumption rate is kept below a certain strain-specific threshold value called critical growth rate (μ_{crit}).

Respiro-fermentative growth

Even under fully aerobic conditions, a mixed respiro-fermentative metabolism is observed when the sugar concentration in the growth medium exceeds a threshold value (typically 1 mM (Verduyn et al., 1984) and the growth rate is higher than so-called critical growth rate (Petrik et al., 1983; Postma et al., 1989). In glucose-limited chemostat and A-stat cultures have been reported the critical growth rates (μ_{crit}) for different strains of *S. cerevisiae* at which respiro-fermentative metabolism sets in (Table 1).

Table 1. Critical growth rate (μ_{crit}) of *S. cerevisiae* strains at which the respiro-fermentative growth set in

Strain	μ_{crit}, h^{-1}	Cultivation mode	Reference
S. cerevisiae ALKO743	0.25	A-stat	Paalme et al., 1997
S. uvarum CCMI 885	0.21	A-stat	Albergaria et al., 2000
S. cerevisiae CEN.PK 113-5D	0.26	A-stat	Olsson et al., 2000
S. cerevisiae DS 28911	0.28	Chemostat	Van Hoek et al., 2000
S. cerevisiae CEN.PK 113-7D	0.30	Chemostat	Van Hoek et al., 1998
S. cerevisiae CEN.PK 113-7D*	0.23	Chemostat	Van Hoek et al., 1998

* Pyruvate decarboxylase overproducing strain

At respiro-fermentative growth, glucose degradation proceeds via two pathways under conditions of aerobic ethanol formation – part is metabolised oxidatively and part reductively, with ethanol being the end product of reductive metabolism (Käppeli, 1986). Pyruvate decarboxylase (PDC, EC 4.1.1.1) converts pyruvate to acetaldehyde, which then reduced to ethanol by alcohol dehydrogenase (ADH, EC 1.1.1.1) with NADH as the electron donor (Fig. 1). Compared to the respiratory consumption of glucose a smaller amount of energy is gained per glucose molecule consumed. The typical values of growth yield based on glucose consumption (Y_{XS}) of *S. cerevisiae* for respirative and fermentative growth are 0.49 (Verduyn et al., 1991) and 0.1 g dwt g^{-1} (Käppeli, 1986; Fiechter and Seghezzi, 1992), respectively, for respiro-fermentative growth those values remain between 0.14–0.18 g dwt g^{-1} (Rieger et al., 1983; Käppeli, 1986; Björkqvist et al., 1997; Hoek et al., 2000).

It was shown that the saturation of the respiratory capacity caused respiro-fermentative growth and ATP is produced simultaneously by fermentation and by respiration (Rieger et al., 1983; Sonnleitner and Käppeli, 1986). Growth of yeast cells on excess glucose leads to an overflow reactions on the level at pyruvate as a consequence of a saturated oxidative capacity (Pronk et al., 1996, Paalme et al., 1997b). An alternative explanation for the occurrence of aerobic alcoholic fermentation at high specific growth rates is the competition of respiration and fermentation for pyruvate (Rieger et al., 1983; Urk et al., 1989) i.e. between pyruvate dehydrogenase and pyruvate decarboxylase. Isolated mitochondria and purified pyruvate dehydrogenase exhibit a much lower K_m values for pyruvate than does pyruvate decarboxylase (Pdc) (Krezse et al., 1981; Urk et al., 1989; Postma et al., 1989).

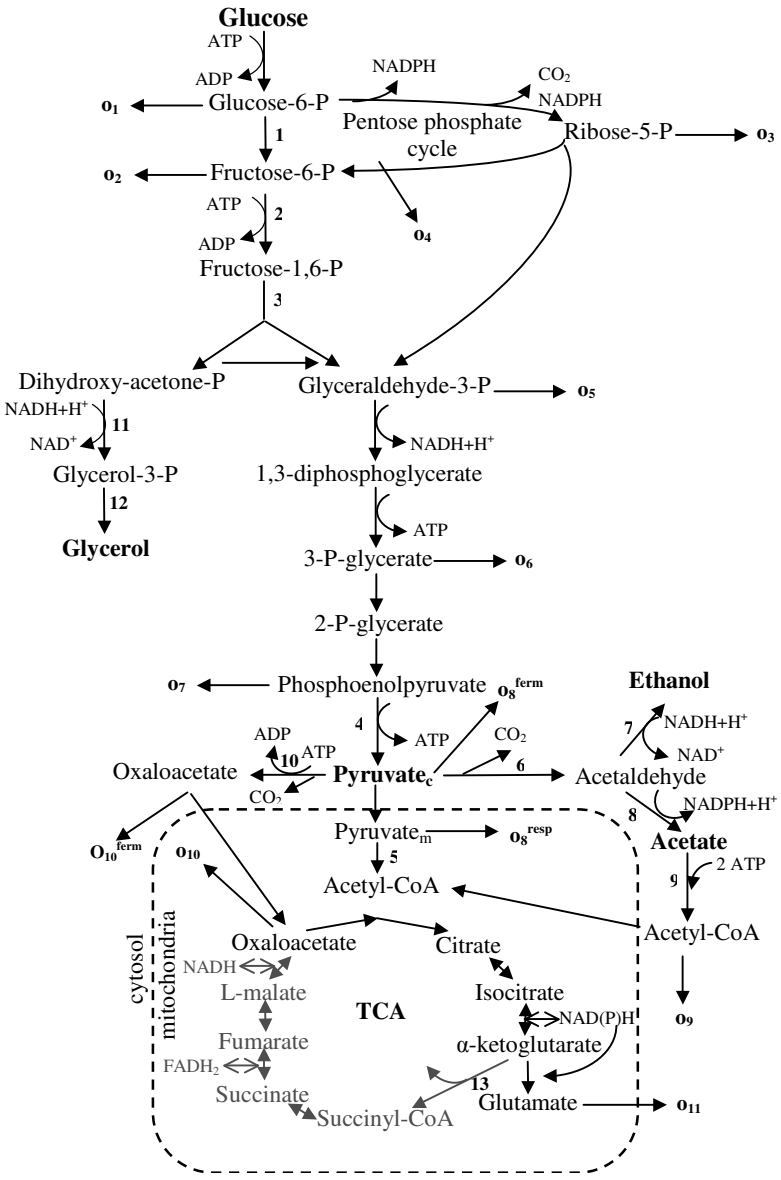


Fig. 1. Simplified scheme of glucose and pyruvate metabolism in *S. cerevisiae*; Key enzymes: 1 – phosphoglucose isomerase, 2 – phosphofructokinase, 3 – fructose-1,6-diphosphatase, 4 – pyruvate kinase, 5 – pyruvate dehydrogenase; 6 – pyruvate decraboxylase, 7 – alcohol dehydrogenase, 8 – acetaldehyde dehydrogenase, 9 – acetyl-CoA-synthase, 10 – pyruvate carboxylase, 11 – glycerol-3-phosphate dehydrogenase, 12 – glycerol-3-phosphatase, 13 – isocitrate dehydrogenase; o_1–o_{11} –fluxes leading to the biomass components. Modified from Fig. 1 (Paper IV) and Paalme et al. (2006)

Hoek et al. (1998) showed that in the Pdc-overproducing strain of *S. cerevisiae* the dilution rate at which aerobic fermentation set in decreased from 0.30 h^{-1} in the reference strain to 0.23 h^{-1}, although the specific respiration rate reached a maximum above the dilution rate at which aerobic fermentation occurred, indicating that a limited respiratory capacity was not responsible for the onset of aerobic fermentation in the Pdc-overproducing strain.

Fermentative growth

Glycerol formation in addition of ethanol, during anaerobic or aerobic conditions at excess of substrate when the respiration is catabolically repressed, help maintains a cytosolic redox state (balance the NADH production during fermentative growth and therefore is conducive to sustain glycolytic catabolism) (Oura, 1977; Albers et al., 1996; Björkqvist et al., 1997). Glycerol is formed as a by-product during ethanol fermentation by yeasts in concentrations of 2.5–3.6% of the produced ethanol (Petrovska et al., 1999). Glycerol formation at fermentative growth is explained by the necessity to consume the NADH formed in cell synthesis, most notably, amino acid synthesis (Nordström, 1966). Also the formation of some organic acids, such as acetic and succinic acid, leads to production of NADH, which is reoxidized in the glycerol formation pathway (Blomberg and Adler, 1992). Glycerol is formed by the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate concomitant with NADH oxidation by NAD^+-dependent glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) Glycerol 3-phosphate is then dephosphorylated to glycerol by glycerol 3-phosphatase (EC 3.1.3.21) (Fig. 1).

The metabolic shift from respiro-fermentative to fermentative growth was accompanied by complex changes of carbon flux throughout the whole central metabolism. The metabolic shift involved an increased flux through pyruvate carboxylase, the fermentative pathways, a flux decrease through the TCA cycle, and a partial relocation of alanine biosynthesis from the mitochondrion to the cytosol (Gancedo, 1998; Frick and Wittmann, 2005). Although the oxidative citric acid cycle cannot proceed, the various intermediary substances of the cycle must be formed to enable a biosynthesis of glutamic acid and the TCA cycle operates as a bifurcated pathway to sustain biomass precursor requirements (Gombert et al., 2001; Blank and Sauer, 2004). During fermentation the NADPH supply under fermentative conditions involves significant contribution of sources other than the pentose phosphate pathway such as e.g. NADPH specific acetaldehyde dehydrogenase or isocitrate dehydrogenase (Frick and Wittmann, 2005).

During fermentative growth, ATP is produced at substrate level phosphorylation, which is stoichiometricaly well fixed and therefore the amount of ATP produced is equal to differences between the concentrations of metabolites formed with net production of ATP (mainly ethanol), and those requiring a net input of ATP (mainly glycerol) as suggests Verduyn et al. (1990a).

1.3. Effect of environmental conditions on the growth of *Saccharomyces cerevisiae*

During growth microbial populations adapt to function optimally in the current environment. Any substantial deviation from the optimum conditions inflicts stress on an organism. The extent of the change will determine whether the organism is killed, ceases growth, or has an increased lag time and reduced growth rate (Ray, 1986; Russell et al., 1995). In nature and in industrial processes, microorganisms encounter a constantly changing environment. In order to survive, they have to able to sense and properly respond to threatening conditions like low and high temperatures, availability of nutrients an oxygen, low pH, osmolarity and presence of inhibitory compounds. These responses to stress tend to be complex and cause changes in growth and metabolic fluxes and rates.

Effect of ethanol

Among environmental stresses that yeast can experience, ethanol toxicity is a major stress, especially encountered in fermentation process. *S. cerevisiae* is capable of very rapid rates of glycolysis and ethanol production under optimal conditions, producing over 50 mmol of ethanol per h g of cell protein (Ciriacy and Breitenbach, 1979). However, this high rate is maintained for only a brief period during batch fermentation and declines progressively as ethanol accumulates in the surrounding broth (Cartwright et al., 1986) around 2% by volume (Dombek et al., 1986). Stressful ethanol levels (>4–6% volume by volume) can decrease yeast growth rates, cell viability, inhibit several systems for nutrient transport (Alexandre and Charpentier, 1998), reduce their metabolic activity, and extreme cases, result in cell death (Martini et al., 2004). Since the addition of ethanol to yeast cells in batch and in chemostat cultures causes a dose-dependent inhibition of ethanol production (Cartwright et al., 1986; Ciriacy and Breitenbach, 1979), most investigations have focused on ethanol as an inhibitor of glycolytic activity. Dombek et al. (1987) showed that added ethanol inhibit fermentation, but the removal of this added or accumulated ethanol does not immediately restore fermentative activity supposing that the decline in metabolic rate in batch culture is due to physiological changes (including possible ethanol damage) rather than the direct effect on glycolytic enzymes.

Although ethanol toxicity in yeast has a rather complex mechanism, the main target of such stress seems to be cell membrane (D'Amore et al., 1990). The result may be an increase in membrane fluidity, a decrease in its structural integrity (Takahasi et al., 2001; Betz et al., 2004) with an accompanying loss in its permeability barrier (Marza et al., 2002). It has been reported that membrane disordering resulting from ethanol exposure leads to dissipation of the electrochemical gradient across the cytoplasmic membrane and changes in transport rates, leakage of intracellular compounds, including enzymatic cofactors and ions essential for cell growth and fermentation (Salgueiro et al., 1988), an increase in hydrogen ion flux across the plasma membrane and changes in internal pH (Leão et al., 1984; Dombek et al., 1987) causing

inactivation of glycolytic enzymes, for examples hexokinase (Alterthum et al. 1989) Although the ethanol enhanced the leakage of protons (Cartwright et al., 1986), the internal pH of the cells was maintained near neutrality up to 12% of ethanol (Dombek et al., 1987), indicating that such leakage must offset by the action of hydrogen ion pumps such as ATPase. Compared with other microorganisms, *S. cerevisiae* appears to be the most alcohol-resistant organism since it is able to grow in the presence of 8 to 12 % (v/v) ethanol and survive exposure to up to 15% (v/v) (Ingram and Buttke, 1984).

It has been reported the in-vivo activation of *S. cerevisiae* plasma membrane H^+-ATPase by ethanol during ethanol-stressed cultivation or following the rapid incubation of cells with ethanol (Monteiro et al., 1994; Monteiro and Sá-Correia, 1998). The H^+-ATPase activation under stress constitutes a response that presumably helps the cell to counteract the stress-induced dissipation of the proton motive force across the plasma membrane and the decrease in the intracellular pH (Viegas et al., 1995; Carmelo et al., 1997). Factors that affect ethanol tolerance beside the activity of ATPase include the proportion of ergosterol in the cellular membranes, the degree of unsaturation of membrane fatty acids (Swan and Watson, 1999), biosynthesis of heat shock-like proteins (Piper et al., 1995), superoxide dismutase, and the capacity of a strain to produce trehalose (Sharma, 1997). Recent studies have shown that the presence of 5 % ethanol in the growth medium caused the induction of Hsp12p and that Hsp12p protected the yeast cell against the deleterious effects of ethanol (Sales et al., 2000).

Effect of temperature

Most laboratory and industrial yeast generally grow best in range of 20–30°C and for *S. cerevisiae* strains maximum growth temperature (T_{max}) range from 35–43°C (Walker, 1997). Factors affecting the capacity of yeast to survive at high temperatures include the presence of stress-response pathways to signal induction of appropriate heat-shock proteins (Hsp). Several Hsps have been shown to perform molecular 'chaperoning' functions in the yeast (e.g. Hsp 60) preventing protein aggregation and the accumulation of aberrant proteins or assisting in the degradation of stress-damaged proteins by enhancing the flow of substrates through proteolytic pathways (Watson, 1990; Piper, 1993). In *S. cerevisiae* an essential protein is Hsp104, which is important in acquisition of thermotolerance (Sanchez and Lindquist, 1990). Piper et al. (1995) have shown that in yeast a sub lethal heat and ethanol exposure induce essentially identical stress responses causing similar changes to plasma membrane protein composition, reducing the levels of plasma membrane H+-ATPase protein and inducing the plasma membrane-associated Hsp30. In addition to the induction of Hsps following heat shock, yeast cells also respond by accumulating other putative protective compound such as trehalose (Wiemken, 1990; Simola et al., 2000), glycogen and glycerol (Omori et al., 1996). Trehalose is thought to act as a thermoprotectant by stabilizing cell membranes and increasing the temperature stability of yeast cellular proteins (Iwahashi et al., 1995)

Increase in temperature cause increase in membrane fluidity and therefore the permeabilization of membranes, particularly the plasma membrane lead to the dissipation of the transmembrane H^+ gradient and to a decrease in intracellular pH (Watson, 1987; Weitzel et al., 1987; Swan and Watson, 1999). Viegas et al. (1995) have shown that during exponential growth at temperatures of 30–39°C, the specific activity of H^+-ATPase in plasma membrane of *S. cerevisiae* increased with increase in growth temperatures and the optimal temperature for *in vitro* ATPase activity was 42°C. Although the activity of the H^+-ATPase increase with increase in the temperature a decline in the H+-ATPase content in the plasma membranes of *S. cerevisiae* cells that had been exposed to no lethal heat shock was observed (Panaretou and Piper, 1992; Viegas et al., 1995). Activation of the plasma membrane H+-ATPase of yeast cells that have been exposed to sub lethal heat stress might increase the capacity of cells to counteract heat damage. This physiological response has been also observed when the H+ gradient was dissipated and intracellular acidification was induced by the membrane active toxic metabolites as ethanol and octanoic acid (Monteiro et al., 1994; Salgueiro et al., 1988; Viegas et al., 1998). It have been showed that the activation of the plasma membrane H+-ATPase or pre accumulation of heat shock proteins due to stress conditions (for example exposure to ethanol and low pH or mild heat pre-treatment) establish enhanced thermotolerance in yeasts (Coote et al., 1991, 1994; Piper, 1995; Hallberg et al., 1996; Lindquist and Kim, 1996; Guyot et al., 2005).

Effect of oxygen

Although *S. cerevisiae* is regarded as a facultative anaerobe, oxygen is required for the synthesis of two essential cellular constituents, sterols and unsaturated fatty acids (Andreasen et al., 1954; Rogers and Stewart, 1973; Kirsop, 1974; Jakobsen and Thorne, 1980). Thus, in anaerobic conditions the unsaturated fatty acids and sterols should be to add to the growth medium to ensure the growth of yeast (Rogers and Stewart, 1973). In an attempt to quantitative the oxygen requirements for lipid biosynthesis in yeast cells, Rogers and Stewart (1973) have calculated apparent K_m value of 0.3 μM oxygen for the synthesis of ergosterol. David and Kirsop (1973) found that synthesis of sterols during a classic fermentation, as well as the oxygen requirement of the yeast cells, are yeast strain dependent. For biosynthetic requirements the maximum oxygen uptake rate of 0.04–0.14 mmol g dwt^{-1} h^{-1} for different strains of *S. cerevisiae* have been reported (Depraetere et al., 2003). Thus, the biosynthetic oxygen requirements of facultatively fermenting yeast are small, compared to the maximum values of the specific respiratory rate which remaining in the range of 6–9 mmol g dwt^{-1} h^{-1} (Fiechter and Seghezzi, 1992; Paalme et al., 1997b; Hoek et al., 1998).

The biosynthesis of essential membrane lipids, such as sterols and (unsaturated) fatty acids, an adequate cellular oxygen supply is essential. Indeed, low levels of these compounds give rise to an alteration of the yeast membrane structure and, consequently, membrane-linked biochemical processes do not function optimally (Aries and Kirsop, 1978). It has been reported that membrane disordering resulting in from the limitation of oxygen leads in

leakage of intercellular compounds and an increase in hydrogen ion flux across the plasma membrane and changes in internal pH. Furthermore, an optimised oxygen supply is critical, because too much oxygen causes yeast degeneration because of the toxic effect of oxygen radicals (Clarkson, 1991).

Effect of osmolarity

Since water is absolutely essential for yeast growth, any external conditions which result in reduced water availability to cells (addition of high amounts of sugar or salt to a growing culture) will impair yeast growth and metabolism. *S. cerevisiae* is regarded as non-osmotolerant yeast. The most effective osmoregulatory compatible solutes in yeast are polyols, particularly glycerol (Blomberg and Adler, 1992). The cytosolic concentration of glycerol is controlled by a plasma membrane channel (encoded by *FPS1* gene), which opens and closes depending on the absence and presence, respectively, of hyperosmotic stress (Luyten et al., 1995). In *S. cerevisiae*, transcriptional control elements, termed the stress response elements (STREs), are activated by wide variety of stress conditions (including low pH, nutrient starvation, oxidative stress osmotic shock, etc.). It is now known that a signalling system called the *HOG* (high osmolarity glycerol) pathway (Albertyn et al., 1994; Rep et al., 1999) controls osmotic regulation of transcription via the stress response element (STRE) (Schüller et al., 1994).

Another metabolite, the disaccharide trehalose, accumulates during salt adaptation (Sharma, 1997). Trehalose accumulates in the cells in response to a number of stress conditions, and it has been shown that trehalose reduces protein aggregation and maintains polypeptide chains in a partially folded state, thus facilitating their refolding by cellular chaperones and help to maintain membrane integrity in stress conditions (Singer and Lindquist, 1998; Wiemken et al, 1990). However, trehalose does not accumulate under salt stress to levels that would have a major impact in the osmotic properties of the cells (Ölz et al., 1993); in this regard the increased amount of glycerol seems by far the most important factor (Blomberg, 2000) Thomas et al. (1994) have found that other compounds such as amino acids may also at as osmoprotectant during very high-gravity fermentation by *S. cerevisiae.*

Effect of pH

Most *S. cerevisiae* strains grow at pH values of 2.5–8.5, and the kinetics of growth and fermentation are not affected between pH 3.5 and 6.0 because of the tight control of intracellular pH (Walker, 1997). Generally, extracellular pH variations have limited impact on yeast cytosolic pH unless the medium comprises weak organic acids. Unlike undissociated lipid-permeable weak acids, which can diffuse freely through the cell membrane and ionise in the cell to yield protons, that acidify the interior of the cell (Krebs et al., 1983; Holyoak et al., 1996), strong acids lower the external pH, but are not able to permeate through the cell membrane. Strong acids exert their antimicrobial effect by denaturing enzymes present on cell surface (Carmelo et al., 1996) and by lowering the cytoplasmic pH due to increased proton permeability when the pH

gradient is very large. Growth may limited by a reduction in the activity of ion transport system in which case essential ions and nutrient will not be taken up (Booth and Kroll, 1989).

The ability of yeast cells to grow at high external hydrogen ions concentration reflects their capacity to maintain control over their internal pH by excluding hydrogen ions. Intracellular pH is regulated to within relatively narrow ranges in growing cells (at pH around 6 in *S. cerevisiae*; Arneborg et al., 2000), mainly through the action of plasma membrane proton-pumping ATPase (H^+-ATPase). This membrane enzyme plays an important role in yeast physiology; it is creates the electrochemical proton gradient that drives the uptake of nutrients by secondary transport (Serrano, 1988) and implicated in intracellular pH homeostasis and influence tolerance to stress factors such as heat (Coote et al., 1994, Viegas et al., 1995) ethanol (Rosa et al., 1991; 1996), organic acids at low pH (Holyoak et al., 1996; Viegas et al., 1998) and deprivation of nitrogen source (Benito et al., 1992). It is generally accepted that the H^+-ATPase in the plasma membrane of yeast cells is activated when posed to low pH. This idea comes from the results of Eraso and Gancedo (1987) who reported the increase in plasma membrane ATPase activity when yeast cells were incubated in a buffer at pH 3.5 acidified by succinic acid as compared to one at pH 6.5. No activation was found when HCl instead of succinic acid was used to acidify the incubation medium to the same low pH (Carmelo et al., 1996). Carmelo et al. (1996) shows in the experiments with *S. cerevisiae* that the content of the ATPase protein in plasma membrane was similar across the pH range of 2.5–6.0 (HCl as acidulant was used), however at lower pH (above 2.5) the ATPase activity was 30% of the maximal value observed at pH 6.0 and growth was impaired. The decrease of plasma membrane ATPase activity in cells grown at low pH was partially accounted for by its *in vitro* stability, which decreased sharply at pH below 5.5, although the reduction of activity *in vivo* was far below the values expected from *in vitro* measurements (Carmelo et al., 1996).

Effect of weak organic acids

Weak organic acids (e.g., acetic, propionic, sorbic and benzoic acids) are used in food processing because of their antimicrobial activity. At the pH below 4 most of the molecules of these organic acids are undissociated and able to diffuse through the cell plasma membrane and enter the cytosol. In the cytosol the pH is neutral and acids dissociate, reducing the intracellular pH (pH_i) below the normal physiological range tolerated by the cell and thus inhibiting cell growth (Krebs et al., 1983; Holyoak et al., 1996). It has been shown that weak organic acids in the culture medium induce a specific pattern of gene expression required for optimal stress response (the transport of anions and protons out of the cell) (Piper et al., 2001). Yeasts have a well-developed system for intracellular pH homeostasis, dependent preferably upon a proton-translocating plasma membrane protein H^+-ATPase (Viegas and Sà-Correia, 1991; Holyoak et al., 1996; Carmelo et al., 1997). Adaptation of *S. cerevisiae* to the environment containing water-soluble organic acids has been shown to require also high activity of the ATP-binding cassette (ABC) transporter Pdr12 in the

plasma membrane, which catalyses active efflux of acid anions from the cytosol (Piper et al., 1998; Holyoak et al., 1999, 2000). Without an active efflux process the charged acid anions cannot readily diffuse out of the cell and will accumulate in acid-stressed cells. The protective systems, proton and anion pumping lead to the depletion of cellular ATP. The efflux pumps need at least two ATP molecules per each organic acid molecule entering the cell (Piper et al., 2001). This additional energy requirement is reflected in dramatic decrease in biomass yield and reduction in the growth rate (Warth, 1988; Viegas and Sá-Correia, 1991; Stratford and Anslow, 1996; Piper et al., 1997; Quintas et al., 2005).

Furthermore, it has been reported that the growth of yeasts in the presence of a weak organic acid depends upon optimal glycolytic flux (Holyoak et al., 1996). Krebs et al. (1983) suggest that weak organic acids inhibit glycolysis due to the acidification of the cytosol, i.e. inhibition is exerted mainly at the phophofructokinase (*pfk1*) level. According to Pearce et al. (2001), the growth of yeast cells is probably inhibited by their reduced capacity to generate ATP (due to the inhibition of glycolytic flux), combined with their need to expend considerable amounts of ATP for maintaining homeostasis.

The inhibition mechanisms for short- and medium-chain aliphatic organic acids are different. The short-chain monocarboxylic acids act as "classical weak-acid preservatives", having an inhibitory effect via entering the cells and lowering the intracellular pH after dissociation (Krebs et al., 1983; Brown and Booth, 1991; Carmelo et al., 1997; Arneborg et al., 2000). Medium-chain monocarboxylic acids are described as membrane active substances (Stratford and Anslow, 1996). It is likely that more lipophilic organic acids may significantly affect the spatial organization of the plasma membrane, interfering with its function as a matrix for enzymes and as a selective barrier, thereby leading to the dissipation of the proton motive force across the membrane and to intracellular acidification (Sà-Correia et al., 1989; Stevens and Hofemyer, 1993). Decanoic acid has been reported to increase the passive flow of protons through the plasma membrane and to induce leakage of amino acids from the cells (Sà-Correia et al., 1989; Stevens and Hofemyer, 1993).

1.4. Cultivation strategies in physiological studies

Mainly batch and chemostat cultures have been used in yeast studies. The both of them have their clear limitations and therefore new cultivation methods A-stat, D-stat and auxo-accelerostat have been introduced.

1.4.1. Batch cultivation

Simplicity in process control and operation are considered the main advantages of batch culture and therefore the batch cultivation is the most common cultivation technique in the laboratory practice. In batch culture, all substrates are added excess at the beginning of the cultivation and after the lag phase biomass increases exponentially with maximum growth until the inhibition by primary or secondary metabolites or substrate limitation occurs. Although the temperature, pH and gaseous compound can be kept in a range not affecting the

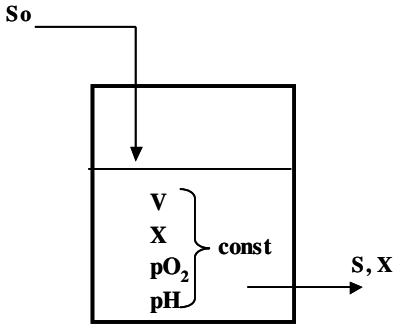
culture characteristics, the growth-associated parameters as substrate, biomass and product concentrations are changing and may affect the values of growth characteristics significantly. Also the inevitable decrease in the glucose concentration due to the growth of *S. cerevisiae* in batch culture can lead to the transition between the different physiological growth states: fermentative, respiro-fermentative and respirative (Käppeli et al., 1986; Fiechter and Seghezzi, 1992).

1.4.2. Continuous cultivation

The continuous cultivation techniques have many advantages compared to the regular batch. It allows keeping the cultivation conditions constant (or in neutral range), choose the optimal biomass density or specific growth rate for experiment and start experiments with a steady-state culture.

Chemostat and turbidostat

The continuous culture methods, chemostat (Monod, 1950; Novic and Szilard, 1950) and turbidostat (Bryson and Szybalski, 1952) were introduced about half a century ago and have proved to be accurate techniques for the determination of culture characteristics in precisely defined steady-state culture conditions. In chemostat, the dilution rate (*D*), and in turbidostat the biomass (*X*) is held constant by manipulating the rate at which medium is fed.



Steady-state:
$dX/dt = 0$
$\mu = D$

Fig. 2. Simplified scheme of the performance of chemostat and turbidostat; S_0; S – substrate concentration in the feeding and outflow medium, D – dilution rate, V – volume, X – biomass concentration

The chemostat cultivation system allows manipulation of the specific growth rate (which at steady-state is equal to the dilution rate) while keeping other important growth conditions constant. Similar to industrial fed-batch cultivation, sugar-limited chemostat cultivation allows fully respiratory growth of *S. cerevisiae* on sugars (von Meyenburg, 1969; Postma et al., 1989; Weusthuis et al., 1994). In case of glucose-limited chemostat culture the fermentative growth in steady-state conditions can be studied only in anaerobic environment. In addition, it is difficult to carry out the chemostat cultivation at maximum growth rate (metabolic activity) of the microorganism. The chemostat has been widely used in studies of effects of specific growth rate (or limiting substrate) on the physiology and growth of *S. cerevisiae* (von Meyenburg, 1969; Postma et al., 1989; Verduyn et al., 1990b; van Hoek et al., 1998, 2000).

The auxostat cultures – modifications of turbidostat 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 using biomass related parameter for feedback control to maintain a desired concentration. In an auxostat, the cultures establish the feeding rate as it is adjusted to match their rate of metabolism (rate of biomass production, oxygen consumption, dioxide production rate, etc.). While the well-known chemostat is stable and simple for investigating continuous cultivation at dilution rates lower than maximum specific growth rate, an auxostat tends to be much more stable at high dilution rates. Population selection pressures in an auxostat lead to cultures that grow rapidly.

The pH-auxostat is beside the turbidostat the most widely applied type of auxostat. The pH change is often an excellent indication of growth and meets the requirements as a growth-dependent parameter as defined by Frederickson et al. (1970). The pH-auxostat couples the addition of fresh medium to pH control. The exact cause of pH change varies among organisms. It represents the summation of the consumption and production of different ionic species. For example, actively growing yeast acidifies their growth medium, through a combination of different ion uptake, direct secretion of organic acids and CO_2 evolution.

Chemostat and auxostat are, however, time and medium-consuming as 4–5 culture volume are needed to obtain the steady state (Tempest et al., 1967) and several steady state points are required to obtain the environment (T, pH, pO_2, etc.) response growth curve. Stepwise changes in dilution rate or environmental conditions in chemostat experiments can cause oscillation (Fiechter and von Meyenburg, 1968) or premature washout of culture (Paalme et al., 1995). In common chemostat and auxostat studies one cultivation parameter is changed stepwise while the others are kept constant or in the range not affecting the growth characteristics.

Novel chemostat based cultivation methods

A-stat is the modification of chemostat – continuous culture with smooth change in desired growth rate (Paalme and Vilu, 1992; Paalme et al., 1995). Like in a chemostat, a steady state culture is obtained, after that the dilution rate (D) is increased with a constant acceleration rate a (h^{-2}) according to the control algorithm: $D(t) = D_0 + a*t$, where D_0 is the dilution rate at the initial steady-state (h^{-1}), and t is the running time. This cultivation procedure enables a fast screening of cell behaviour under a wide range of limiting substrate concentrations, providing reliable data of microbial physiology and determination of culture characteristics in shorter time than the classical chemostat technique (Paalme et al., 1997a). Although the A-stat will not provide the steady-state growth conditions the quasi-steady-state at slow acceleration rates can be maintained. The results of this method may even better characterize the behaviour of the culture in nature and also in biotechnological processes than chemostat does, because in the natural ecosystem the smooth change in growth conditions is quite common, the steady-state conditions being rather the exception than rule (Paalme et al., 1995). The A-stat method has been

successfully applied for the physiological studies of *Escherichia coli* (Paalme et al., 1995, 1997a), *S. cerevisiae* (Paalme et al. 1997b), *Lactobacillus plantarum* (Kask et al., 1999), *Saccharomyces uvarum* (Albergaria et al., 2000) and *Zygosaccharomyces rouxii* (Sluis et al., 2001).

D-stat is also a modification of chemostat. Like in chemostat the dilution rate is kept constant and one of the environmental parameter was changed at constant rate. This method is effective for detection of the critical values of environmental or cultivation factors. D-stat method has been used for the study of physiology of *S. uvarum* (Nisamedtinov et al., 2003). The A-stat and D-stat suited first of all for studies of growth in substrate-limited conditions, however, in environment with excess of essential substrates, neither of them was satisfactory.

In this study, a novel auxostat based cultivation method with smooth change in environmental conditions, auxo-accelerostat**,** was developed to map the effect of environmental conditions on the fermentative growth of *S. cerevisiae*

AIMS OF THE STUDY

The aims of the present study were:

1) to develop and evaluate the auxostat-based continuous cultivation method, which would enable to study the effect of environmental conditions on the growth of a microorganism at excess of growth substrates;

2) to study the effect of smoothly changing environmental conditions on the fermentative growth of *S. cerevisiae* S288C by quantifying key growth characteristics (specific growth rate, specific ATP production rate, growth yield);

3) to determine the critical values of environmental parameters leading to a growth inhibition of *S. cerevisiae* and to map the cellular physiological response to the changing environmental conditions.

MATERIALS AND METHODS

2.1. Yeast strain and cultivation conditions

The laboratory yeast strain *Saccharomyces cerevisiae* S288C kindly provided by Dr. Matti Korhola (Alko Ltd., Finland) was used in the experiments. In all auxo-accelerostat experiments, glucose containing (50 g l^{-1}) mineral medium was used and the exact composition of the medium is given in Paper I and IV. In A-stat experiments the glucose concentration in mineral medium was 10 g l^{-1}.

During the auxo-accelerostat cultivation the temperature was maintained at 30°C, dissolved oxygen concentration (pO_2) within the range of 2.5–5.0% of air saturation and pH at 3.6 by titration with 1M NH_4OH. The relatively low cell densities (0.6–0.8 g dwt l^{-1}) were chosen to avoid the growth inhibition by metabolites or any other growth-related factors. Yeast inoculum was grown in the glucose-supplemented (50 g l^{-1}) mineral medium in batch culture overnight.

2.2. Cultivation system and cultivation process routines

The fermentation equipment from Applikon (The Netherlands) and Bioengineering (Switzerland) was combined and used for the auxo-accelerostat experiments. The principal scheme of the computer-controlled cultivation system employed in the experiments is shown in Paper II (Fig. 1). The cultivation system was equipped with pH, pO_2, and temperature sensors as well as CO_2 analysers for the exhaust gas analysis. Two variable speed pumps were used for the feeding of the growth medium and for variations of its composition. In addition, two fixed speed pumps for the alkali titration and culture removal were used. The culture volume was kept constant (760 or 170 ml) by means of a level indicator or balance linked to the outflow pump. The total volume of the outflow was quantified off-line or on-line by determining the weight of the out flowing culture medium. Headspace aeration was applied to supply the system

with the oxygen required for the synthesis of the sterols and unsaturated fatty acids and to measure the CO_2 production in auxostat experiments. The aeration was controlled by changing the airflow into the fermenter or by changing the stirrer speed. The pH of the culture was controlled either by titration with 1M NH_4OH or by addition of fresh cultivation medium in pH-auxostat experiments. The whole system was linked to PC through AD/DA interface, ADI-1030 Biocontroller (Applikon, The Netherlands) and the cultivation control software "BioXpert" (Applikon, the Netherlands), a commercial version of "FermExpert" (Vinter et al. 1992) was used for the cultivation control and analysis of the growth characteristics. Parallel to on-line measurements of temperature, pH, pO_2 and CO_2, the concentration of biomass, glucose, glycerol and ethanol were measured off-line. Samples for analyses were taken from the effluent line at 0.5 h interval.

The auxo-accelerostat experiments were carried out in pH-, pO_2- and CO_2-control mode, i.e. the pH of the culture, dissolved oxygen concentration of air saturation or CO_2 concentration in the outflow gases, respectively, were the growth-related set point parameters for the feedback regulation of the feeding to keep the pH, pO_2 or CO_2 at the desired level. The culture vessel was inoculated with overnight batch-wise grown culture and cultivated in batch mode until the desired biomass concentration was obtained. Then the continuous cultivation in auxostat mode was initiated. The simple control algorithm, using two-level control of the feeding rate (F) was used: $F = V*D_{LOW}$ or $V*D_{HIGH}$, where V is the culture volume and D is the dilution rate. D_{HIGH} was chosen equal to about $1.5*\mu_{max}$ and D_{LOW} to $0.3*\mu_{max}$. In case of pH- or pO_2-auxostat, if either the pH or pO_2 value was higher than the given set point value (pH_{set} or pO_{2set}), the lower dilution rate (D_{LOW}) was applied. On contrary, when pH or pO_2 was lower than the set-point value, the higher dilution rate (D_{HIGH}) was applied, respectively. In case of CO_2-auxostat the converse algorithm was applied (IF $CO_2>CO_{2set}$ $F=V*D_{HIGH}$ ELSE $F=V*D_{LOW}$). However, if needed, the set-point values of D_{LOW} and D_{HIGH} were corrected manually during the experiments when μ was approaching to D_{LOW} or D_{HIGH} values.

At first a steady-state culture (constant biomass, μ, Q_{ATP}, Y_{ATP}) was obtained (usually after four or five culture volumes had passed through the fermenter). After that the computer-controlled smooth change with constant rate of change in certain environmental factor was started – auxo-accelerostat cultivation mode**,** and respective culture response growth curves were recorded. All the auxo-accelerostat experiments were started when the culture was in steady state conditions; consequently the growth-characteristics (μ, Q_{ATP}, Y_{ATP}) in the starting point of all experiments were similar as described in the Papers III and IV.

2.3. Calculation of growth characteristics

Growth characteristics of yeast cells were calculated with "BioXpert" software on the basis of culture optical density (OD), removal rate of culture broth (pmp_{out}) and main metabolites (ethanol and glycerol) as follows (the values of the off-line parameters are measured as described in Papers II, III and IV):

$$\mu = \frac{pmp_{\text{out}}}{V} + \frac{\mathrm{d}(OD)}{\mathrm{d}t(OD)} \quad (1)$$

$$Q_{\text{eth}} = \frac{eth(pmp_{\text{out}})}{V(OD)\gamma} + \frac{\mathrm{d}(eth)}{\mathrm{d}t(OD)\gamma} \quad (2)$$

$$Q_{\text{glr}} = \frac{glr(pmp_{\text{out}})}{V(OD)\gamma} + \frac{\mathrm{d}(glr)}{\mathrm{d}t(OD)\gamma} \quad (3)$$

$$Y_{GE} = \frac{Q_{glr}}{Q_{eth}} \quad (4)$$

$$Q_{\text{ATP}} = Q_{\text{eth}} - Q_{\text{glr}} \quad (5)$$

$$Y_{\text{ATP}} = \frac{\mu}{Q_{\text{ATP}}} \quad (6)$$

where μ – specific growth rate (h^{-1}), Q_{eth} and Q_{glr} – specific ethanol and glycerol production rates (mmol g dwt^{-1} h^{-1}), Q_{ATP} – specific ATP production rate (mmol ATP g dwt^{-1} h^{-1}), Y_{ATP} – growth yield based on ATP production (g dwt mol ATP^{-1}), V – current fermenter volume (ml), pmp_{out} – removal rate of culture broth (ml min^{-1}), OD – optical density at 540 nm, γ – strain specific biomass conversion factor, *eth* and *glr* – ethanol and glycerol concentration in the fermenter (mM).

For the calculation of Q_{ATP} in Paper IV the following formula is used:

$$Q_{\text{ATP}} = Q_{\text{eth}} - Q_{\text{glr}} + B \cdot \mu \quad (7)$$

where Q_{eth} and Q_{glr} are specific ethanol and glycerol production rates (mmol g dwt^{-1} h^{-1}), μ is specific growth rate (h^{-1}), B is variable characterizing the biomass composition (mmol ATP g dwt^{-1}). The calculation of B is described in the Paper IV.

RESULTS AND DISCUSSION

3.1. Growth of *S. cerevisiae* S288C in A-stat, batch and auxostat culture (Papers II and IV)

The A-stat cultivation technique was used to study the effect of the change in the dilution rate on the growth of *S. cerevisiae* S288C and to determine the critical growth parameters (μ_{crit}, S_{crit}) leading to transitions from respirative to respiro-fermentative and fermentative growth. A-stat experiments with smooth increase in the dilution rate at constant acceleration rate (a) of 0.022 h^{-2} (Paper II) and 0.01 h^{-2} (unpublished data) were performed.

In A-stat culture (a = 0.01 h^{-2}) the dilution rate was increased from the steady-state value of 0.15 h^{-1} to 0.46 h^{-1}. In the dilution rate range of 0.15–0.18 h^{-1} the biomass concentration was almost constant and the dilution rate was equal to the specific growth rate (μ=D). Neither acetate nor ethanol accumulated, indicating that the metabolism of glucose was fully respirative.

Above the dilution rate of 0.18 h^{-1} A-stat culture showed a sharp decrease in biomass concentration and specific growth rate. Acetate and one hour later ethanol started to accumulate in the culture medium (Fig. 3).

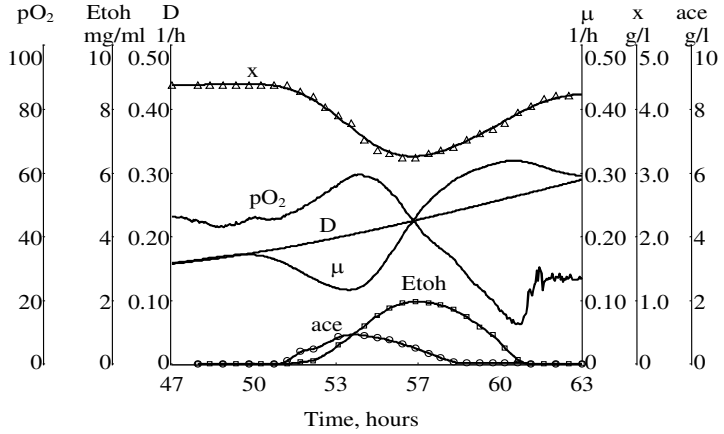


Fig. 3. A-stat culture of *S. cerevisiae* S288C (a=0.01 h^{-2}) at the dilution rate (D) of 0.15–0.30 h^{-1}; Etoh, ace, x – concentration of ethanol, acetate and biomass, pO_2 – concentration of dissolved oxygen (%), μ–specific growth rate

Later, despite the increase in the dilution rate, acetate and ethanol disappeared from the culture media. Turning the production of overflow metabolites (acetate and ethanol) back into consumption shows that the yeast cells are able to adjust their respiratory metabolism for growth at higher specific glucose consumption and growth rates. At the dilution and growth rate of 0.32 h^{-1} ($\mu_{crit}^{respirative}$) first acetate and then ethanol began to accumulate again. Differently from the first metabolic switch, the decrease in the specific growth rate was not observed (Fig. 4).

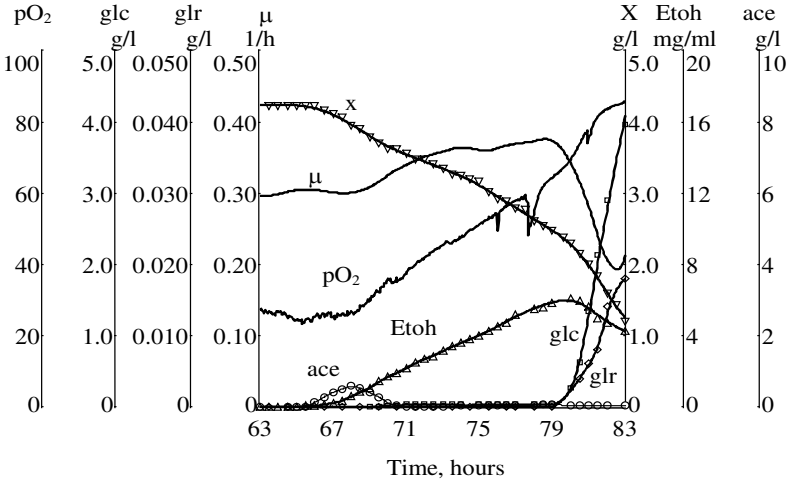


Fig. 4. A-stat culture of *S. cerevisiae* S288C (a=0.01 h^{-2}) at the dilution rate of 0.30–0.46 h^{-1}; Etoh, ace, glc, glr, x – concentration of ethanol, acetate, glucose, glycerol and biomass, pO_2 – dissolved oxygen concentration (%), μ – specific growth rate

Acetate disappeared later, however, the accumulation of ethanol continued. Increase in pO_2 and decrease in biomass concentration indicate the exhaustion

of the respiratory capacity and onset of respiro-fermentative growth. During respiro-fermentative growth the specific growth rate, in parallel to the dilution rate increased up to the value 0.37 h^{-1} (μ_{crit}^{ferm}). Then, with the start of glucose accumulation the specific growth rate decreased and glycerol started to accumulate. The formation of glycerol is required to balance the NADH production during fermentative growth. The transitions from one growth mode to another cause rearrangements in cell metabolism and therefore the decrease in the specific growth rate occurred at the onset of catabolic repression and the culture became unstable. Although there were signs of increase in the specific growth rate at the end of the experiment, the maximum specific growth rate remained far below that obtained in batch and auxostat culture.

The maximum values of the specific growth rate (μ_{max}) 0.43±0.02 h^{-1} obtained in the three parallel batch experiments with different glucose concentrations (30, 50, 70 g l^{-1}) (Paper IV) and auxostat cultures at steady-state conditions (glucose concentration 50 g l^{-1}) (Papers III, IV) were higher than the maximum growth rate of 0.37 h^{-1} obtained during respiro-fermentative growth in A-stat culture. The glycerol to ethanol formation ratio (Y_{GE}) 0.1±0.01 mol mol^{-1} recorded for batch and auxostat experiments corresponds well to the ratio calculated theoretically from the stoichiometric model of fermentative growth (Paper IV) and suggests that the repression of respiration was complete. In batch and auxostat culture at steady-state conditions the maximum specific ATP production rate of *S. cerevisiae* S288C was 35.6±1.1 mmol g dwt^{-1} h^{-1}, being about 1.5 times higher than the corresponding values obtained for different strains of *S. cerevisiae* (Ciriacy and Breitenbach, 1979; Hoek et al., 1998). In batch culture the yeast cells grew at a maximum specific growth rate (and maximum specific ATP production rate) up to the biomass concentration of 0.8 g l^{-1}, and then started to decrease; also the Y_{GE} decreased (Fig. 2a, Paper IV). Differently from batch culture in auxostat experiments, the biomass concentration can be kept at optimal range (supporting maximum growth and production rate) for a long period.

4.2. Auxostat based cultivation technique, auxo-accelerostat (Papers I-V)

To study the effect of environmental conditions on the fermentative growth and metabolic rates of *S. cerevisiae*, a novel auxostat based cultivation technique, auxo-accelerostat, was developed, introducing computer-controlled smooth changes in selected environmental conditions for quantitative growth characterization. Like in accelerostat (A-stat), the auxo-accelerostat experiments were started in steady state culture with respect to constant biomass concentration and dilution rate. The growth characteristics of steady-state cultures (T=30°C, pH=3,6, pO_2=2.5%, X< 0.8 g l^{-1}) of 20 auxo-accelerostat experiments (experiments are described in Papers III and IV) varied only a little: specific growth rate (μ) was 0.44±0.01 h^{-1}, specific ATP production rate (Q_{ATP}) was 35.6±1.1 mmol g dwt^{-1} h^{-1}, growth yield based on ATP production (Y_{ATP}) was 12.4±0.2 and the ratio of glycerol to ethanol production rate (Y_{GE}) was 0.1±0.01. Thus a similar starting point (steady-state culture) was obtained for all the auxo-accelerostat experiments.

Unlike A-stat culture, the auxo-accelerostat culture operates at the excess of essential substrates (enabling study of the fermentative growth of *S. cerevisiae*) and instead of the dilution rate the desired environmental factor E (T, pH, or pO_2, etc.) is smoothly changed at a constant rate of change (dE_i/dt=constant). The auxo-accelerostat cultivation mode is best suited for culture characterization at maximum growth rates.

The auxo-accelerostat method developed is unique in that it enables studies of the effect of the change rate of environmental or cultivation conditions on culture behaviour, which is important for control of industrial processes. The CO_2-auxo-accelerostat experiments with an increase in the concentration of monocarboxylic acids clearly demonstrated that different rates of change may affect the growth of yeast differently (Paper III). The auxo-accelerostat, as well as the A-stat cultivation technique, enables studying the microbial response (adaptation) to smoothly changing environment. The growth characteristics and growth response curves produced by experiments with a smooth change in environmental conditions (auxo-accelerostat culture) can differ from the values obtained using the ″classical″ auxostat culture in which the culture parameter changed step-wise and the steady state conditions were obtained.

In pH-, pO_2 and CO_2-accelerostat experiments a growth-dependent parameter for feedback control was applied to maintain a desired concentration of biomass. The results of the experiments showed that smooth change in the environmental parameter can affect the biomass concentration (Fig. 5, Paper I; Fig. 7, Paper V) (due to the changes in media buffering capacity, oxygen transfer rate, CO_2 production rate, etc.). The changes in biomass concentration can affect the culture characteristics, as shown in batch culture (Fig. 2a, Paper IV). Therefore, in selecting the auxo-accelerostat method and experimental conditions, a possible change in biomass concentration should be minimized. For example, the pH-auxo-accelerostat mode is not suitable for the study of the effect of weak organic acid salts, because of changes in the buffering capacity of the medium and therefore also in the biomass concentration.

4.3. Effect of changing environmental conditions on the fermentative growth of *S. cerevisiae* S288C (Paper IV)

The effect of pH, pO_2, T, and concentration of biomass, ethanol, propanol, NaCl, yeast extract and tryptone on the growth and metabolic rates of *S. cerevisiae* was studied using pH-, CO_2- and pO_2-auxo-accelerostat culture with smooth increase in the given environmental parameter. The main results of these studies are presented in Paper IV. Some important results will be discussed below.

The effect of biomass

The results of the batch experiments showed that once the biomass concentration reached a level of 0.8 g dwt l^{-1}, the specific growth rate (μ) and the specific ATP production rate (Q_{ATP}) started to decrease (a sharp decrease occurred at the biomass concentration of 1.2 g l^{-1}) (Fig. 2a, Paper IV). As this effect could be due to fast changes in culture conditions during batch

cultivation, the effect of biomass concentration was studied also in pH-auxo-accelerostat culture with controlled increase in the biomass concentration. The biomass concentration in pH-auxo-accelerostat was increased at an about twofold slower rate (0.1 g l^{-1} h^{-1}) than in the exponential growth phase of batch culture, to give more time for the adaptation of cells to growth at higher cell densities. Nevertheless, like in batch culture, the specific growth rate (μ) remained constant (maximal) only up to the biomass concentration of 0.8 g dwt l^{-1} and began to decline together with the ATP production rate (Q_{ATP}) at higher cell densities (Fig. 2b, Paper IV). Therefore, to avoid the effect of biomass concentration on the growth characteristics, all the following auxo-accelerostat experiments were carried out at biomass concentrations below 0.8 g dwt l^{-1}.

The effect of alcohols: ethanol and propanol

The biomass concentration itself, for example due to "Quorum sensing", seems unlikely to be responsible for the decrease in the specific growth rate and specific ATP rate (inhibition of ethanolic fermentation). The accumulation of fermentation products such as ethanol or organic acids would be expected to affect adversely the specific growth rate. The effect of exogenous ethanol on the fermentative growth of *S. cerevisiae* S288C was studied in three pH-auxo-accelerostat experiments by increasing its concentration in the growth medium at rates of 3.1, 4.1 and 6.6 g l^{-1} h^{-1}, respectively. Low initial biomass concentration (0.1–0.15 g dwt l^{-1}) was used in order to minimize the effect of inherent metabolites (including ethanol) produced by the yeast cells while the steady-state culture was obtained. With the increase in ethanol concentration the specific growth rate started to increase, obtaining the maximum value at exogenous ethanol concentrations of 5–12 g l^{-1} (Fig. 3a, Paper IV). At ethanol concentrations higher than 10–12 g l^{-1} the specific growth rate decreased.

However, with increase in biomass concentration the specific growth rate started to decrease already when the biomass and ethanol concentrations in the growth media were 0.8 g dwt l^{-1} and 3–4 g l^{-1}, respectively (Fig. 2a, Paper IV). Thus, the factor(s) causing the decrease in the specific growth rate of *S. cerevisiae* S288C at biomass concentrations higher than 0.8 g dwt l^{-1} should be other(s) than the accumulation of ethanol in the growth media. The fact that external ethanol is much less toxic than fermentatively derived ethanol has been pointed out also previously. Jones (1988) has suggested that toxic by-products can contribute to the apparent toxicity of endogenously produced ethanol.

The effect of added ethanol on growth yield (Y_{ATP}) and specific ATP production rate (Q_{ATP}) could not be determined due the problems with determining separately the added ethanol and ethanol produced by the yeast cells. To predict the effect of the added ethanol on Y_{ATP} and Q_{ATP}, the pH-auxo-accelerostat experiment with smooth increase in propanol concentration was carried out (Fig. 5). The inhibition of the specific growth rate and Q_{ATP} was observed at the concentration of 1 g l^{-1}, which was about 10-fold lower than in the experiments with added ethanol. Growth yield (Y_{ATP}) remained constant up to the propanol concentration of 4 g l^{-1} and then started to decrease, while the decline in Q_{ATP} stopped and even increased at higher concentrations. The

concentration of propanol causing a 50% reduction in the specific growth rate was 15 g l^{-1}, being about 3-fold lower than in case of added ethanol (respective value was 43 g l^{-1}). At the propanol concentration of 28 g l^{-1} the growth rate decreased down to the value of 0.13 h^{-1}.

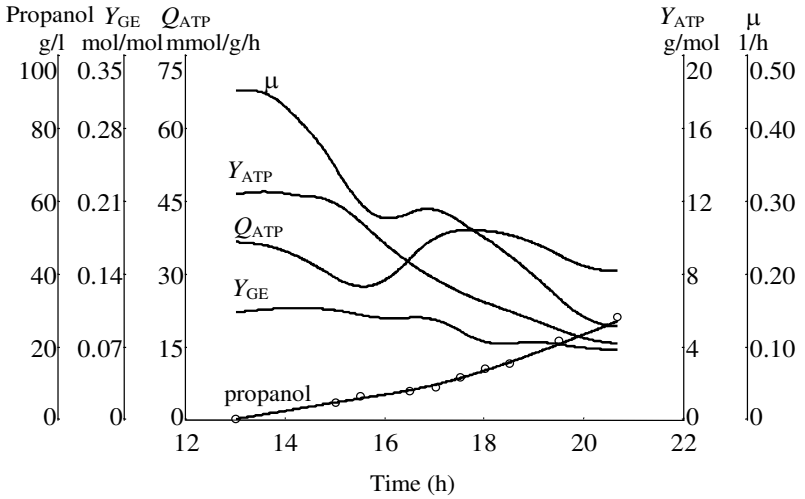


Fig. 5. The effect of propanol concentration on the growth of *S. cerevisiae* S288C in pH-auxo-accelerostat culture; μ - specific growth rate, Y_{ATP} – growth yield based on ATP production, Q_{ATP} – specific ATP production rate, Y_{GE} – glycerol to ethanol production ratio

The results of the experiment suggest that lower concentrations of propanol (< 4 g l^{-1}) inhibit the growth rate through the decrease in Q_{ATP}. At higher concentrations of propanol the leakage of cell membranes may occur causing increase in maintenance energy and decrease in growth yield. A direct effect of a lower propanol concentration on glycolytic machinery would be unlikely, as the specific ATP production rate increased with further increase in propanol concentration higher than 6 g l^{-1}.

The effect of pH

The specific growth rate, specific ATP production rate and growth yield were almost constant during smooth decrease in culture pH from 3.6 down to 2.8 in CO_2-auxo-accelerostat experiment. Further decrease in the culture pH down to 2.3 caused a decrease in the growth yield (Y_{ATP}) and specific growth rate, but not in the specific ATP production rate (Q_{ATP}). Obviously, the energy requirement for the maintenance of the intracellular homeostasis increased sharply and led to a decrease in growth yield. It has been shown that the culture pH of 3.2 affects the kinetics of growth and fermentation of *S. cerevisiae*. In this study the specific ATP production rate was still high at the pH of 2.3 (Fig.6). Stable Q_{ATP} suggests that the intracellular pH remained in the range not affecting the glycolytic capacity. It has been shown, that the ability of yeast cells to grow at high concentration of external hydrogen ions reflects their

capacity to maintain control over their internal pH by excluding hydrogen ions, mainly through the action of plasma membrane H^+-ATPase.

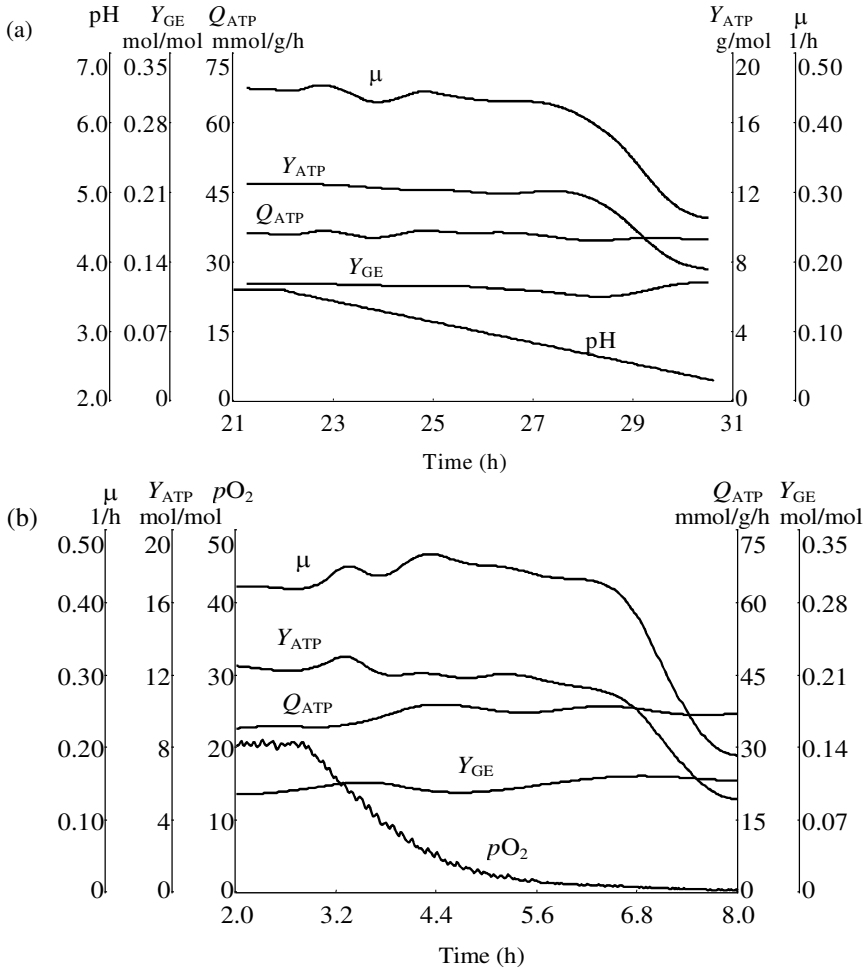


Fig. 6. The effect of smooth decrease in (**a**) culture pH and (**b**) dissolved oxygen concentration (%) on the fermentative growth of *S. Cerevisiae,* μ – specific growth rate, Y_{ATP} – growth yield based on ATP production, Q_{ATP} – specific ATP production rate, pH – pH of culture medium, pO_2 – dissolved oxygen concentration of air saturation (%), Y_{GE} – ratio of glycerol to ethanol production.

The effect of oxygen

The pH- and pO_2-auxo-accelerostat approach was used to study the effect of dissolved oxygen concentration on the growth of *S. cerevisiae* S288C. In both experiments the specific growth rate was not significantly affected by changing the dissolved oxygen concentration, at least in the range of 20% to 0.8% of air saturation at 30°C. Significant decrease in both the growth yield and specific growth rate, but not in the specific ATP production rate, occurred at oxygen concentrations below of 0.8% of air saturation (Fig. 6).

These results suggest that the transition to oxygen limitation as well as to low pH values exert a decreasing effect on yeast growth rate mainly due to decrease in Y_{ATP} (increase in maintenance energy). It has been reported that the membrane disordering resulting from the limitation of oxygen leads to leakage of intracellular compounds and an increase in proton efflux and changes in internal pH. Based on our data, the intracellular pH has to stay in the range supporting the glycolysis (Q_{ATP} remains almost constant).

The effect of salinity

The pO_2 and pH-auxo-accelerostat approach was used to study the effect of salt concentration on the growth of *S. cerevisiae* S288C. During smooth increase in NaCl concentration in the culture medium (in both experiments) the specific growth rate remained constant up to the NaCl concentration of 13 g l^{-1} (Fig. 7). At higher NaCl concentrations, the growth rate started to decrease along with the Q_{ATP} values, and at the NaCl concentration of 39 g l^{-1} the specific growth rate was 0.13 h^{-1} (the NaCl concentration causing a 50 % reduction in the specific growth rate was 31 g l^{-1}). Growth yield remained constant up to NaCl concentrations of 27 g l^{-1} and sharply decreased at higher concentrations. This suggests that no significant energy consumption for maintaining the cell homeostasis took place at NaCl concentrations lower than 27 g l^{-1}. The glycerol to ethanol formation ratio (Y_{GE}) decreased from 0.11 mol mol^{-1} to 0.05 mol mol^{-1} when the NaCl concentration was increased up to 12–16 g l^{-1}.

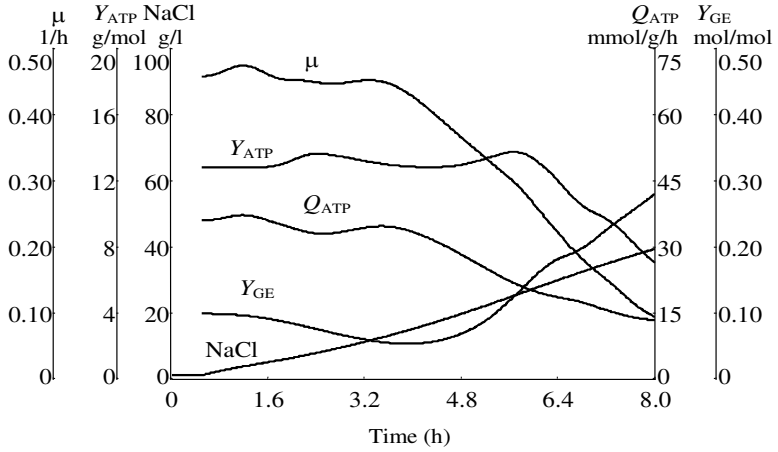


Fig. 7. The effect of NaCl concentration on the growth of *S. cerevisiae* S288C in pO_2-auxo-accelerostat culture; μ - specific growth rate, Y_{ATP} – growth yield based on ATP production, Q_{ATP} – specific ATP production rate, Y_{GE} – glycerol to ethanol production ratio

With further increase in the NaCl concentration, Y_{GE} began to increase, reaching the value of 0.28 mol mol^{-1} at the NaCl concentration of 39 g l^{-1}. The increased glycerol to ethanol formation ratio suggests that the adaptation of yeast cells to osmotic pressure involves the rearrangement of intracellular metabolic fluxes. The increase in the glycerol to ethanol formation ratio at higher NaCl concentrations should involve reactions leading to the additional NADH formation.

The growth inhibiting effect of NaCl on the yeast cell was probably different from that induced by the transition to low pH or pO_2 limitation. Indeed, in case of NaCl, the decrease in the specific growth rate was caused primarily by the decrease in Q_{ATP}, while in case of low pH and oxygen limitation the decrease in the growth rate was caused by the decrease in growth yield. The high concentrations of extracellular NaCl may directly affect the rate of glucose transport and therefore also the specific ATP production rate (Q_{ATP}). On the other hand, the accumulation of intracellular glycerol as the response to the increasing osmolarity through the HOG signalling pathway (Albertyn et al., 1994; Rep et al., 1999; Blomberg, 2000; Wuytswinkel et al., 2000) can significantly alter the maximum velocities of ATP requiring biosynthetic reactions and lead to a decrease in the specific ATP production rate through the feedback mechanism.

The effect of temperature

In general, the specific growth rate of *S. cerevisiae* increased with increase in temperature up to the optimal highest temperature (T_{max}). The effect of the increase in the cultivation temperature on the growth of *S. cerevisiae* S288 was studied in the pH-auxo-accelerostat experiment by smoothly increasing the temperature from 25 to 45°C (Fig. 6, Paper IV). Due to the increase in temperature, the specific growth rate as well as the specific ATP production followed the Arrhenius equation up to 34°C (Fig. 8) (1/T=0.00326 corresponding to 34 °C).

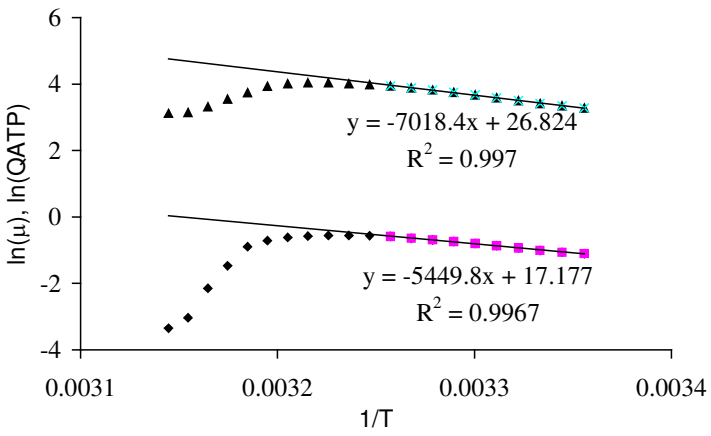


Fig. 8. Arrhenius plots for specific growth rate and specific ATP production rate versus temperature (K^{-1}), ▲– ln (Q_{ATP}) ■ – ln (μ)

The specific growth rate obtained a maximum value of 0.57 h^{-1} at temperature range of 34–35°C and started to decrease quickly as the temperature exceeded the value 38°C (T_{max}). It can be assumed that the sharp decline was caused by

rapid denaturation of enzymes, which are essential to growth. Growth yield (Y_{ATP}) decreased from 13 to 10 g dwt mol ATP^{-1} almost linearly as the temperature increased from 25 to 40°C, and decreased sharply with further increase in temperature. The lower growth yield indicates that growth becomes energetically more costly with increasing growth temperature. It has been shown that the specific activity of H^+-ATPase in the plasma membrane of *S. cerevisiae* increases with increase in growth temperature (Viegas et al., 1995). The decrease in growth yield (increase in maintenance energy) can be explained also by the stress response - synthesis of heat shock proteins, degradation and replacement of denaturated proteins, etc. The glycerol to ethanol formation ratio increased with increasing temperature up to the value of 0.13 mol mol^{-1}. Omori et al. (1996) has been reported the accumulation of glycerol due to elevated growth temperature.

The effect of tryptone and yeast extract

The effect of tryptone was studied in two pH-auxo-accelerostat experiments by increasing the tryptone concentration in the growth medium to 1.3 g l^{-1} (Fig. 9) or 5 g l^{-1}, respectively. Due to the increase in the tryptone concentration in the growth medium, the specific growth rate increased in parallel with growth yield (Y_{ATP}) and obtained the maximum value of 0.62 h^{-1} (Y_{ATP}=18.7 g mol^{-1}) at the tryptone concentration of 0.5–0.9 g l^{-1} (Fig 9).

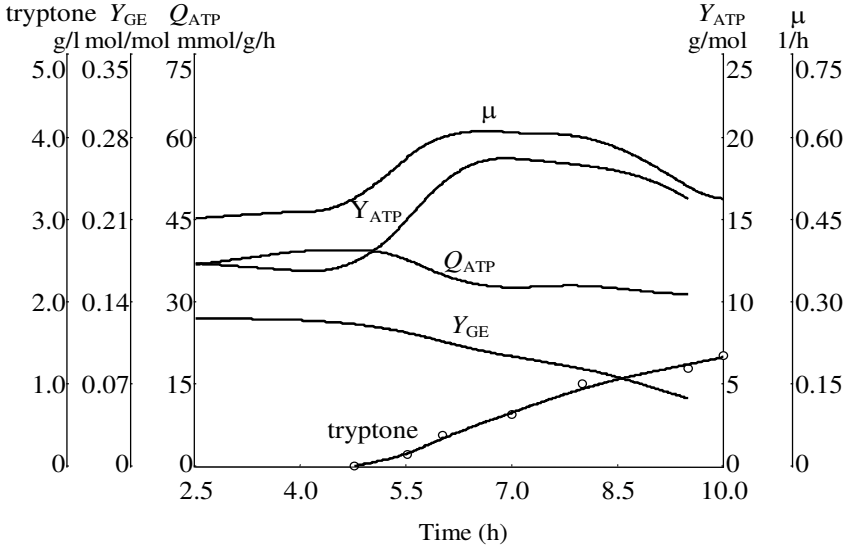


Fig. 9**.** The effect of tryptone on the growth of *S. cerevisiae* S288C in pH-auxo-accelerostat culture; μ – specific growth rate, Y_{ATP} – growth yield based on ATP production, Q_{ATP} – specific ATP production rate, Y_{GE} – glycerol to ethanol production ratio

Unlike growth yield (Y_{ATP}), the specific ATP production rate (Q_{ATP}) decreased with the increasing tryptone concentration in the growth medium. In the experiment where the tryptone concentration was increased to 5 g l^{-1}, the maximum growth rate was 0.61 h^{-1}. In both experiments tryptone

concentrations higher than 1 g l^{-1} had an inhibitory effect on the specific growth rate.

In the experiment with increasing the yeast extract concentration, the specific growth rate increased and obtained a maximum value of 0.56 h^{-1} at the yeast extract concentration of 1.5 g l^{-1} (Fig. 7b, Paper IV). In contrast to tryptone addition, yeast extract had a stimulating effect both on the glycolytic activity (Q_{ATP} increased) and growth yield. The Y_{ATP} value was 14.5 g mol^{-1} at the yeast extract concentration of 1.5 g l^{-1} (16% higher than without yeast extract). Growth yield (Y_{ATP}) increased from 9 to 11.5 g mol^{-1} with increasing the concentration of yeast extract up to 4.5 g l^{-1}.

The addition of tryptone or yeast extract decreased the glycerol to ethanol production ratio (Y_{GE}). This may be explained by partial replacement of endogenously synthesized amino acids by exogenous ones, leading to decrease in Y_{GE} due to the decrease in the intracellular NADH formation through the corresponding amino acid synthesis reactions.

4.4. Effect of short- and medium-chain monocarboxylic acids on the growth of *S. cerevisiae* S288C (Paper III)

The CO_2-auxo-accelerostat approach with smooth increase in the concentration of added monocarboxylic acids was used to study the effect of aliphatic monocarboxylic acids (formic, acetic, propionic, valeric, octanoic and decanoic acids) on the growth and metabolic rates of *Saccharomyces cerevisiae* S288C. In the experiments with formic, propionic and decanoic acids different change rates of acid concentrations were applied to study the effect of rates of change itself. The actual rates of change of the concentrations of monocarboxylic acids in the fermenter are given in Paper IV (Table 1). The toxic effect of each monocarboxylic acid on the growth of *S. cerevisiae* 288C was characterized and quantified from the respective dose-effect curves as IC_{50} value (mM) using two different endpoints: a decrease of 50% in the specific growth rate ($IC_{50\mu}$) and a decrease of 50% in the growth yield on produced ATP basis (IC_{50YATP}). The main results of these studies are presented in Paper III. Some important results will be discussed below.

Smooth and slow increase in the concentration of aliphatic monocarboxylic acids formic, acetic, propionic (water-soluble), valeric and more lipophilic octanoic and decanoic acids resulted in rapid decrease in the growth yield (Y_{ATP}) and specific growth rate (μ), however the specific ATP production rate (Q_{ATP}) increased or stayed almost constant (Fig. 1, 3 and 4, Paper III). The Q_{ATP} increased by 4–81% depending on the type of the acid (Table 2, Paper III)). With further increase in formic, acetic, propionic and valeric acid concentration the decrease in Y_{ATP} and μ stopped and the growth of yeast cells continued for a while at an almost constant rate. The plateau was obtained when Y_{ATP} had decreased by 40–60% of the initial value and the resistance to the higher acid concentration had apparently developed**.** The resistance to decrease in Y_{ATP} can be explained by the decreasing flux of weak acids into the cell, which is proportional to the chemical gradient of the undissociated acid, diffusion

coefficient and surface area of the yeast cell walls. Probably, the diffusion of weak acid through the cell wall and membrane decreased due to the changes in membrane fatty acid composition or in the cell envelope. No sign of adaptation processes and development of resistance to higher acid concentration were recorded in the experiments with octanoic and decanoic acids (Fig. 4, Paper III).

The concentrations of formic, acetic, propionic, valeric, octanoic and decanoic acids causing the 50% reduction in the specific growth rate ($IC_{50\mu}$) were, respectively, 18.1, 47.1, 33.6, 2.3, 0.16 and 0.07 mM (Fig. 10). The octanoic and decanoic acids was more toxic to yeast cells than shorter-chain monocarboxylic acids, having 150–300 times lower $IC_{50\mu}$ values than for formic, acetic and propionic acids. The decrease of 50% in growth yield based on ATP production (IC_{50YATP}) was observed at lower formic, acetic, propionic, valeric and octanoic acids concentrations (13.9, 32.7, 23.5, 1.7 and 0.13 mM, respectively) than that in the specific growth rate (the IC_{50YATP} value of decanoic acid was similar to that of $IC_{50\mu}$) (Fig. 10).

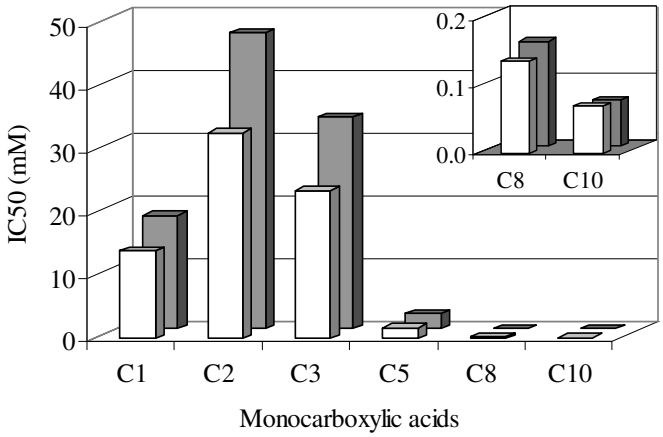


Fig. 10. Concentrations of undissociated monocarboxylic acids (mM) that caused 50% reduction in growth yield based on ATP production (□ – IC_{50YATP}) and specific growth rate (■ – $IC_{50\mu}$) in CO_2-auxo-accelerostat experiments with a slower rate of change of the acid concentration (Table 1, Paper III); the smaller graph in the top right-hand corner is magnification of the IC_{50} values for octanoic and decanoic acids

The higher sensitivity of ATP-based growth yield than growth yield could be explained by increase in the specific ATP production rate compensating the increased energetic expenditures (pumping out of protons and anions) for ATP-dependent efflux of the added acids. When the concentration of formic, propionic or decanoic acid was increased at a 1.5-3 times higher rate, than in experiments with a slower rate of change of acid concentration (Table 1, Paper III), the Q_{ATP} decreased not increased. Therefore, the $IC_{50\mu}$ values were notably lower (up to 5-fold) in case of a more rapid increase in the concentration of acid in the medium (Fig. 11). Thus, the toxic effect depends not only on the nature of the monocarboxylic acid (lipophilicity), but also on the rate at which its concentration changes in the growth environment.

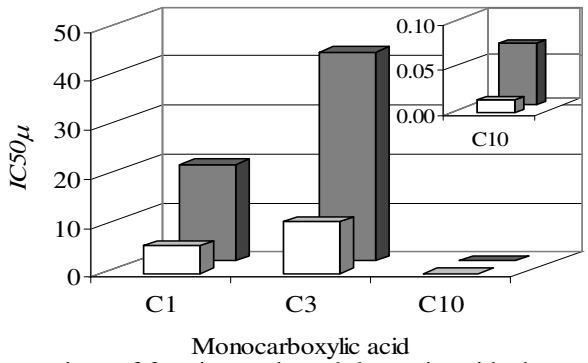


Fig. 11**.** Concentrations of formic, acetic and decanoic acids that caused 50% reduction in specific growth rate ($IC_{50\mu}$) (■ – slower and □ – faster rate of change, Table 1 in Paper III); the smaller graph in the top right-hand corner is magnification of the IC_{50} value for decanoic acids

The results of the CO_2-auxo-accelerostat experiments showed the relationship between the rate of change of the concentration of a monocarboxylic acid and inhibitory pattern of the specific growth rate. In case of a *slower rate* of change in the acid concentration the decrease in the specific growth rate can be fully explained by the decrease in growth yield, whereas the specific ATP production rate increased (or remained constant) (Fig. 12).

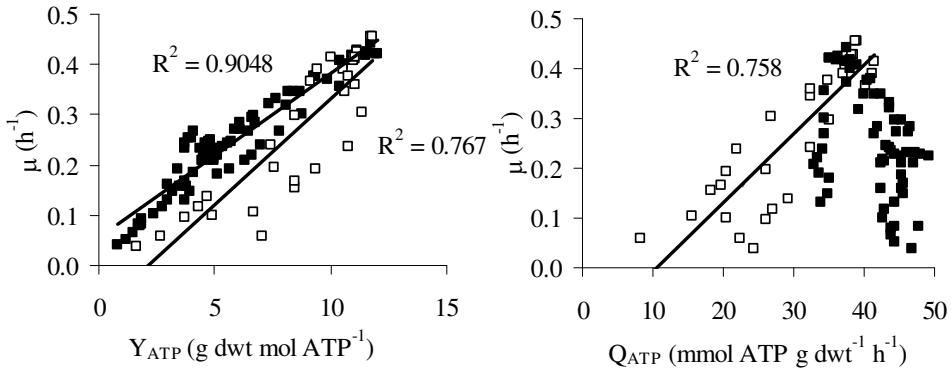


Fig. 12. Relationship between **(a)** the specific growth rate (μ) and growth yield (Y_{ATP}) and **(b)** specific growth rate and specific ATP production rate (Q_{ATP}) of *S. cerevisiae* S288C at slower (■) and faster (□) rate of change in the concentration of monocarboxylic acids (formic, acetic, propionic, valeric, octanoic and decanoic acid)

Evidently, slow increase in acid concentration did not lead to the decrease in the intracellular pH (pH_i) large enough to inhibit the glycolysis and leave sufficient time for the induction of the protective system (the membrane proteins H^+-ATPase and Pdr12). The *faster rate* of change of the acid concentration in the growth environment caused decrease in the specific growth rate due to the decrease in the specific ATP production rate and growth yield (Fig. 12). Obviously, rapid increase in acids concentration in the growth environment caused acidification of the cytosol, which led to inhibition of glycolysis and decrease in Q_{ATP}.

4.5. Growth rate control (Papers III and IV)

During a smooth change in environmental conditions towards favourable or unfavourable environmental conditions, microorganisms are able to adapt to the new situation by changing their metabolic fluxes and molecular composition.

The results of **A-stat experiments** showed that during the glucose (and energy) limited growth the growth rate was dependent on the concentration of substrate as the growth rate increased in parallel to the increase in the dilution rate. However, starting from a certain dilution rate (D = 0.18 h^{-1}), the overflow metabolism (indicated by the accumulation of acetate and ethanol in the growth medium), accompanied by a sudden decrease in the specific growth rate, was observed. It could be supposed that biosynthetic reactions and/or respiratory system became inhibited by the intracellular accumulation of acetate and decrease in intracellular pH, accompanied by the exhaustion of respiratory capacity. Later, as a result of stress response, the protons and acetate ions were either pumped out from the yeast cells or oxidized inside the cells. In both cases, the induction of enzymes, responsible for efflux or assimilation of acetate (enzymes of the citrate cycle or respiratory chain), is required. Anyhow, in parallel with the decrease in the content of acetate (and ethanol) in the culture medium (due to consumption), the specific growth rate started to increase. At the second critical dilution rate (onset of the respiro-fermentative growth) accompanied by the accumulation of acetate, no decrease in specific growth rate was observed. It is probable that the yeast cells were already adapted to acetic acid stress and the relative change rate (a/D) was smaller than at the first acetate accumulation.

When microorganisms grow in the conditions of excess of substrate, the growth rate is determined mostly by the capacity of catabolic and/or anabolic reactions and energy cost for biomass synthesis and maintenance. In the **auxo-accelerostat experiments,** where *S. cerevisiae* S288C was grown in the conditions of excess of glucose, different varying environmental conditions were studied. These environmental conditions were either unfavourable (for example, the non-optimal pH or dissolved oxygen concentration) or favourable for growth (in case of addition of tryptone that is a source of additional nutrients).

In unfavourable conditions the specific growth rate decreased, and three main patterns for growth inhibition can be outlined.

1. Smooth decrease in culture pH, dissolved oxygen concentration and slow increase in monocarboxylic acid concentration caused a decrease in growth yield (Y_{ATP}). The reduction in growth yield shows that in the stress conditions the expenditure of energy (ATP) for the maintenance of the cell homeostasis is increased. The constant or increasing specific ATP production rate indicates that the glycolytic pathway was activated rather than inhibited in those conditions. In fact, the slow increase in the concentration of water-soluble or lipophilic monocarboxylic acids (formic, acetic, propionic, valeric, octanoic) was accompanied by an up to 80% increase in the specific ATP production rate. It is known that the H^+-ATPase

and ATP-binding cassette (ABC) transporter Pdr12 in the plasma membrane of yeast cells are activated due to the presence of weak organic acids (Holyoak; et al., 1996; Carmelo et al.; 1997; Piper et al., 1998;) and proton and anion pumping out leads to the depletion of cellular ATP. The auxo-accelerostat experiments with a slow change in monocarboxylic acids clearly showed that the complete glycolytic capacity (capacity to produce ATP) reveals itself in stress conditions, which exerts heavy energy load on the yeast cells. Probably, the glycolytic flux in *S. cerevisiae*, at least during fermentative growth, is controlled by the demand for ATP. Koebmann et al. (2002) have proposed a similar concept for regulation of glycolytic activity in *Escherichia coli*.

2. Fast increase in monocarboxylic acid concentration resulted in sharp decline in both specific ATP production rate and growth yield. This suggests that the growth is inhibited by reduced capacity to generate ATP (decline in Q_{ATP}) due to the inhibition of glycolytic flux, combined with the need to expend considerable amounts of ATP on maintaining homeostasis (decrease in growth yield). It is believed that the primary mode of action of weak organic acid is to reduce pH_i below the normal physiological range, leading to growth arrest (Cole et al., 1987; Carmelo et al., 1997). It has been shown that a weak organic acid can inhibit the glycolysis due to the acidification of the cytosol, i.e. inhibition is exerted mainly at the phosphofructokinase level (Krebs et al., 1983). Changes in pH_i are also important in controlling cell cycle and rates of DNA and RNA synthesis in the eukaryotic cells (Madshus, 1988).
3. Slow increase in the concentration of biomass, NaCl and propanol led to a decrease in glycolytic activity (Q_{ATP}), indicating the inhibition of the glycolysis directly or through feedback mechanism(s) coupled to ATP consumption in biosynthetic reactions. The constant value of Y_{ATP} indicates that stress response requiring additional energy for the maintenance of cell homeostasis in these conditions was not induced at the first stage of the stress.

In more **favourable conditions** the specific growth rate increased. As a result of smooth addition of yeast extract or tryptone the specific growth rate increased by 27% and 40%, respectively. The addition of tryptone or yeast extract, accompanied by the decrease in the ratio of glycerol to ethanol production rate (Y_{GE}), indicates partial replacement of endogenously synthesized amino acids by the exogenous ones. The effects of studied environmental conditions on the growth of *S. cerevisiae* have been summarized in a simplified scheme (Fig. 13).

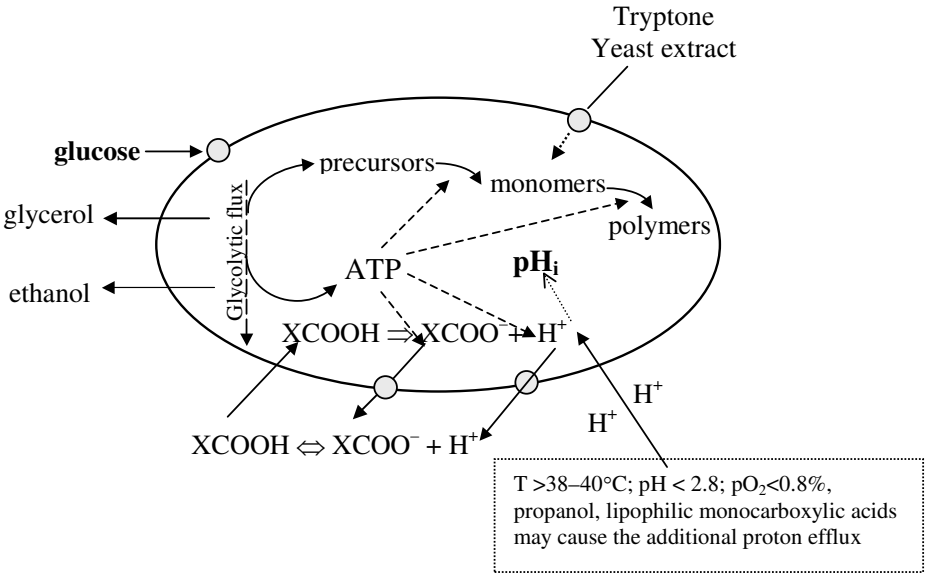


Fig. 13. Simplified scheme of catabolic and anabolic block of the metabolism of *S. cerevisiae* and demand for ATP (dashed arrows) in anabolic reactions and for keeping cell homeostasis

In general, we showed that the specific rate of ATP production, characterizing the net capacity of catabolic reactions, was obviously not a growth rate-limiting step for *S. cerevisiae* in a mineral medium (30°C). Our assumption was based on the fact that slow addition of the monocarboxylic acids to the culture medium (increasing the maintenance expenditures that cells were able to compensate via adaptation mechanisms) was accompanied by up to 80% increase in the specific ATP production rate. Moreover, as the smooth addition of tryptone or yeast extract to the growth medium resulted in the increase of the specific growth rate, showing that anabolic reactions leading to monomer synthesis, and not catabolic ones, were growth rate limiting in these conditions. Anabolic metabolism was shown to be a growth-limiting step also for *E. coli* (Jensen et al., 1990; Koebmann et al., 2002). Still, as the expenditure of energy for keeping cell homeostasis increases (accompanied by decrease in growth yield), the growth rate control shifts into the catabolic block.

CONCLUSIONS

1. A novel auxostat-based cultivation method - auxo-accelerostat - that enables to introduce the smooth and constant rate of change in environmental conditions was developed. It was shown that:
    a. The auxo-accelerostat technique enables to study the effect of environmental conditions on microbial growth and metabolism as well as adaptation mechanisms at excess of growth substrates;
    b. The auxo-accelerostat method is a unique technique as it enables to study the effect of rate of change of environmental conditions on culture behaviour, which is important in nature as well as for the control of industrial processes.
2. A new important environmental factor – the rate of change of environmental parameter - was introduced. The results of CO_2-auxo-acclerostat experiments with added monocarboxylic acids clearly demonstrated that the toxic effect of environmental parameter depends not only the parameter changed (i.e. type of the added monocarboxylic acid) but also on the rate of change of these environmental parameter.
3. Moving smoothly into stress conditions by decreasing the culture pH or dissolved oxygen concentration below optimal and/or by increasing the monocarboxylic acid concentration in the culture medium leads to the growth inhibition mainly due to the increased maintenance costs (growth yield decreased) and not due to the inhibition of ATP production rate.
4. The smooth increase in the concentration of biomass, propanol, and NaCl or rapid addition of monocarboxylic acids into the culture medium leads as first response to the decrease in energy (ATP) production.
5. Auxo-accelerostat experiments showed that in optimal conditions in mineral medium with excess of glucose the capacity of ATP production was obviously not a growth rate limiting step for *S. cerevisiae* and glycolytic activity was controlled by the demand for ATP.
6. The present study demonstrates that the auxo-accelerostat cultivation method, besides being an efficient tool for the quantification of culture characteristics, is also a promising techniques allowing elucidation of the growth rate control mechanism(s) in cells.

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ARTICLE I

Drews, M., Kasemets, K., Nisamedtinov, I., Paalme, T. Continuous cultivation of insect and yeast cells at maximum specific growth rate

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ARTICLE II

Kasemets, K., Drews, M., Nisamedtinov, I., Adamberg, K., Paalme, T. Modification of A-stat for the characterization of microorganisms

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ARTICLE III

Kasemets, K., Kahru, A., Laht, T.-M. and Paalme, T. Study of the toxic effect of the short- and medium-chain monocarboxylic acids on the growth of *Saccharomyces cerevisiae* using CO_2-auxo-accelerostat fermentation system

Submitted: International Journal of Food Microbiology

ARTICLE IV

Kasemets, K., Nisamedtinov, I., Laht, T.-M., Abner, K. and Paalme, T. Behaviour of growth characteristics of *Saccharomyces cerevisiae* S288C in changing environmental conditions: auxo-accelerostat study

Submitted: Antonie van Leeuwenhoek

ARTICLE V

Adamberg, K., Kasemets, K. and Paalme, T. Auxo-accelerostat – a new effective cultivation system for culture characterization

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KOKKUVÕTE

Keskkonnamuutuste mõju uurimine *Saccharomyces cerevisiae* S288 fermentatiivsele kasvule aukso-akselerostaadis

Katsete käigus töötati välja uudne kultiveerimismeetod, aukso-akselerostaat, mis võimaldab uurida mikroorganismide kasvu kontrollitult muutuvates keskkonnatingimustes. Antud meetod on unikaalne, kuna võimaldab uurida toitainete rikkas keskkonnas nii keskkonna parameetrite kui ka nende muutmise kiiruse mõju rakkude metabolismile ja kasvule ning selle läbi uurida ka adaptatsioonimehhanisme. Käesolevas töös rakendati aukso-akselerostaati keskkonna tingimuste mõju uurimisel *Saccharomyces cerevsiae* S288C fermentatiivsele kasvule. Uuriti erinevaid keskkonna parameetreid (s.h. keskkonna happelisus, hapniku kontsentratsioon, temperatuur, monokarboksüülhapped), mis on olulised nii tööstuslike kääritusprotsesside kui ka toiduainete konseveerimistingimuste optimeerimisel.

Aukso-akselerostaat katsetes määrati erinevate keskkonnamuutuste mõju pärmi kasvu erikiirusele (μ), kasvu saagisele (Y_{ATP}) kui ka ATP toomise erikiirusele (Q_{ATP}) ja antud kasvukarakteristikute alusel hinnati vastavate keskkonnaparameetrite toimemehhanisme rakkude kasvule. Näidati, et pärmile ebsoodsates kasvutingimustes, mis oli põhjustatud madalast happesusest, hapniku limitatsioonist või monokarboksüülhapete konsentratsiooni aeglasest suurendamisest kasvukeskkonnas, oli kasvukiiruse langus põhjustatud kasvusaagise langusest. Eelkõige näitab kasvusaagise langus, et rakkudes käivitub antud stressi tingimustes ATP-seoselised kaitsemehhanismid (näiteks membraansed prootonpumbad, jne.). Kuid nii biomassi (ja sellega kaasnevate metaboliitide), propanooli ja NaCl kui ka monokarboksüülhapete kontsentratsiooni kiire suurendamisega kasvukeskkonnas kaasnev kasvukiiruse langus on põhjustatud eelkõige ATP tootmise erikiiruse langusest. Aukso-akselerostaat katsed näitasid selgelt, et monokarboksüülhapete toksilisus sõltub nii nende lipofiilsusest kui ka happe konsentratsiooni muutmise kiirusest, mis põhjustab erinevusi raku metabolismis ja ühtlasi ka adaptatsiooni mehhanismide käivitumises.

Aukso-akselerostaat katsetega näidati, et pärmi *S. cerevisiae* kasvukiirus on optimaalsetes kasvutingimustes eelkõige ära määratud anaboolsete reaktsioonide kiirusega, kuna monokarboksüülhapete sujuval lisamisel kasvukeskkonda suurenes ATP tootmise erikiirus kuni 80%. See näitab, et rakkude glükolüütiline aktiivsus optimaalsetes kasvutinginustes ei ole reguleeritud maksimaalsele võimsusele, samas trüptooni või pärmiekstrakti lisamisel (monomeeride allikas) kasvukeskkonda suurenes kasvukiirus.

Antud katsete tulemused näitavad, et uus läbivoolukultiveerimise meetod, aukso-akselerostaat, on sobiv ja efektiivne nii mikroorganismide kasvukarakteristikute määramisel muutuvates keskkonnatingimustes kui ka kasvuregulatsiooni mehhanismide uurimisel. Antud meetodi alusel on ka võimalik välja töötada uudne *in vitro* test, et määrata ainete toksilisust muutuvates keskkonnatingmustes ja hinnata adaptatiivsete mehhanismide osa ainete toksilisuse moduleerimisel.

Võtmesõnad: *Saccharomyces cerevisiae* S288C, fermentatiivne kasv, kasvukarakteristikud, aukso-akselerostaat, muutuvad keskkonnatingimused, keskkonna parameetrite muutumise kiirus, adaptatsioon

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