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Study of the toxic effect of short- and medium-chain monocarboxylic acids on the growth of *Saccharomyces cerevisiae* using the CO_2-auxo-accelerostat fermentation system

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Abstract

The effect of aliphatic monocarboxylic acids (formic, acetic, propionic, valeric, octanoic and decanoic acids) on the growth and metabolic activity of *Saccharomyces cerevisiae* S288C was studied, using continuous cultivation method — CO_2-auxo-accelerostat with smooth increase in the concentration of added monocarboxylic acids. Slow increase in the concentration of these acids resulted in the 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. On the other hand, Q_{ATP} decreased if the concentration of formic, acetic or decanoic acids was increased rapidly. The toxic effect of aliphatic monocarboxylic acids on the growth of *S. cerevisiae* was characterized and quantified from the respective dose–effect curves as the 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 based on ATP production (IC_{50YATP}). 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. 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. The results of the CO_2-auxo-accelerostat experiments show that 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.

Keywords: *Saccharomyces cerevisiae*; Monocarboxylic acids; Growth characteristics; Toxicity; Stress response; Adaptation; Adenosine triphosphate (ATP); CO_2-auxo-accelerostat; Rate of change

1. Introduction

During growth microbial populations adapt to function optimally in the current environment. Any substantial deviation from the optimum conditions inflicts stress on an organism. Weak organic acids (e.g., acetic and propionic 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 Sá-Correia, 1991; Holyoak et al., 1996; Carmelo et al., 1997). Adaptation of *Saccharomyces 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

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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 a 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 (via 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 (Sá-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 (Sá-Correia et al., 1989; Stevens and Hofemyer, 1993).

Although the behaviour of yeast cultures at the presence of weak organic acids has been studied previously, only a few reports are available on quantitative effects of acids on the growth characteristics in a strictly controlled environment (Brul and Coote, 1999). Little attention has been paid to the effect of the rate of change of weak organic acid concentrations on the specific growth rate and ATP production rate, which are of both practical and theoretical interest. One of the reasons could be the lack of effective cultivation techniques for these studies. In batch culture, the growth-associated environmental parameters (concentrations of biomass, substrate, metabolites, etc.) are changing constantly. 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. Chemostat cultures, as well as A- and D-stat cultures (Paalme et al., 1995; Kasemets et al., 2003), are performed under substrate limitation conditions and therefore are not suitable for the simultaneous study of the effect of weak acids on two growth's parameters (the maximum specific growth rate and growth yield). Thus, we developed a modification of the auxostat, an auxo-accelerostat (Adamberg et al., 2003; Kasemets et al., 2003), which enables the study of the effect of inhibitors (e.g., weak organic acids) at surplus of essential growth substrates in precisely controlled cultivation conditions.

The current paper focuses on the effect of aliphatic monocarboxylic acids (formic, acetic, propionic, valeric, octanoic and decanoic acids) on the growth and metabolic rates as well as other culture characteristics (e.g., μ, Y_{ATP}, Q_{ATP}, $m_{e\Delta}$) of *S. cerevisiae* S288C. The study was conducted in CO_2-auxo-accelerostat with constantly increasing the concentration of the added weak organic acid in the growth environment.

2. Materials and methods

2.1. Yeast strain and cultivation conditions

S. cerevisiae S288C was used in the CO_2-auxo-acelerostat experiments. The exact composition of the mineral medium is described in Paalme et al. (1997). Glucose was used as a carbon source at final concentration of 50 g l^{-1}. Yeast inoculum was grown in the glucose-supplemented (50 g l^{-1}) mineral medium in batch culture overnight. During the CO_2-auxo-accelerostat cultivation the temperature was maintained at 30 °C, dissolved oxygen concentration 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 growth inhibition by metabolites or any other growth factors. In our experiments formic (pK_a 3.75), acetic (pK_a 4.75), propionic (pK_a 4.87), valeric (pK_a 4.84), octanoic (pK_a 4.89) and decanoic acids (pK_a 4.90) are almost undissociated, i.e., in the form of potent growth inhibitors as the pH of the growth medium was kept at 3.6.

2.2. Cultivation system and cultivation process routines

The fermentation equipment from several companies (e.g., Applikon, The Netherlands and Bioengineering, Switzerland) was used. The principal scheme of the computer-controlled cultivation system employed in auxo-accelerostat experiments is described in Drews et al. (1998) and Kasemets et al. (2003). Briefly, the cultivation system was equipped with various speed pumps (feeding and outflow, monocarboxylic acid feeding, etc.), pH, pO_2, CO_2 and temperature sensors, and level and stirrer rate controllers. The whole system was linked through the AD/DA interface, ADI-1030 Biocontroller (Applikon, The Netherlands) and the cultivation control software "BioXpert" (Applikon), a commercial version of "FermExpert" (Vinter et al., 1992), was used. 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. The CO_2 level in the outflow gases was measured by respective analysers. The culture volume was kept constant (780 ml) by means of an overflow tube or a level indicator linked to the outflow pump. The total volume of the outflow was quantified off-line every half an hour.

The experiments were carried out in CO_2-auxostat cultivation mode, i.e. the concentration of CO_2 in the outflow gases was the