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INFLUENCE OF OXYGEN LIMITATION ON GLUCOSE METABOLISM IN *HANSENIASPORA UVARUM* K_5 GROWN IN CHEMOSTAT

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Summary: Growth and metabolite formation were studied as a function of oxygen feed rate, in glucose-limited chemostat cultures of *Hanseniaspora uvarum* K_5 at a dilution rate of 0.26 h^{-1}. Alcoholic fermentation occured at an oxygen feed rate of 80 $mmol.l^{-1}.h^{-1}$. Below this value, pyruvate decarboxylase and alcohol dehydrogenase were present at high levels. In contrast, activities of oxidative metabolism enzymes, pyruvate dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase, decreased.

Introduction

One of the most important parameters with respect to the occurence of alcoholic fermentation in yeasts is the concentration of oxygen in the culture medium (Visser *et al.*, 1990). For example, the Pasteur effect (Lagunas, 1986), the Crabtree effect (Fiechter *et al.*, 1981; Petrik *et al.*, 1983; Käppeli, 1986), the Custer effect (Carrascosa *et al.*, 1981; Wijsman *et al.*, 1984) and the Kluyver effect (Sims et Barnett, 1978) are all closely related to the availability of oxygen.

A few yeast species are able to grow anaerobically. The only one able to grow with specific growth rates higher than 0.10 h^{-1} is *Saccharomyces cerevisiae*. Even in the case of *S. cerevisiae*, anaerobic growth conditions impose additional growth factor requirements (in particular, ergosterol, nicotinic acid and unsattured fatty acids) (Andreasen et Stier, 1953; 1954). *Kloeckera apiculata*, an apiculate yeast found in the first stage of wine fermentation (Lafon-Lafourcade, 1983), exhibited also clear anaerobic growth, with concomitant ethanol production (Visser *et al.*, 1990). Venturin *et al.* (1994) found that a strain (K_5) of *Hanseniaspora uvarum* (perfect state of *K. apiculata*) was also able to grow anaerobically.

This strain, in the presence of oxygen, did not perform alcoholic fermentation, when it was exposed to excess glucose in batch cultivation (Venturin *et al.*, 1994) or in chemostat cultures (Venturin *et al.*, 1995). The limitation of oxygen in this strain has been studied only in partial and strictly anaerobic conditions in Erlenmeyer flasks. Oxygen feed can only be studied as a variable in chemostat cultures when the gas transfer properties of the cultures do not vary substantially among different steady states (Weusthuis *et al.*, 1994).

The aim of the present work was to investigate the function of oxygen in the case of *H. uvarum* K_5, a Crabtree-negative strain. For this purpose, the effect of the oxygen feed rate on glucose metabolism was studied in glucose-limited chemostat cultures. The levels of key enzymes in pyruvate metabolism as well as metabolite production and physiological parameters, such as biomass yield, specific rate of glucose uptake, specific rate of oxygen consumption and carbon dioxide production, were determined.

Materials and methods

Micro-organism: The strain *Hanseniaspora uvarum* K_5 was obtained from the INRA Guadeloupe research station. It was listed as 1-845 in the National Micro-organisms Collection of Institut Pasteur (France).

Chemostat cultivation: Glucose-limited chemostat culture was performed in 2.5 litre fermenter (Applikon) at a dilution rate of 0.26 h^{-1}, temperature of 30°C and air flow rate of 3 $l.min^{-1}$. The culture pH was maintained at 5.0 by automatic addition of 2N NaOH, via an Applikon ADI 1020 biocontroller. The working volume of the culture was kept at 1.5 litre. The dissolved oxygen tension in the culture was regulated by variation of stirrer speed. The mineral medium was prepared as previously described by Venturin *et al.* (1995), with glucose 20 $g.l^{-1}$ in the reservoir medium.

Oxygen limitation experiments: Two types of experiments were carried out.

1) The dissolved-oxygen tension was decreased progressively from 80 to 0 % air saturation by variation of stirrer speed from 1300 to 600 rpm. At every steady-state reached, samples were taken and immediatly centrifuged for the determination of enzyme levels. The determination of extracellular metabolites was performed automatically.

2) After a steady state has been reached at a dissolved-oxygen tension of 50 % air saturation (stirrer speed of 1000 rpm), a shift in dissolved oxygen tension to 0 % air saturation was performed by decreasing the stirrer speed to 600 rpm. Samples were taken at regular intervals (0, 2, 4, 6 and 24 h.) for analysis.

Gas analysis: The exhaust gas flow was measured on line. The oxygen content of the exhaust gas has been determined with a 755A Beckman oxygen analyser and carbon dioxide content with a 870 Beckman infra-red carbon dioxide analyser. Specific rates of CO_2 production (qCO_2, $mmol.g^{-1}.h^{-1}$) and O_2 consumption (qO_2, $mmol.g^{-1}.h^{-1}$) were calculated from these values.

Culture dry weight: Cell growth was monitored by measuring the optical density (O.D.) at 420 nm using a DU7 spectrophotometer. One O.D. unit corresponds to 0.180 ± 0.002 $g.l^{-1}$ dry weight at $D = 0.26$ h^{-1}.

Metabolite analysis: Residual glucose, glycerol, acetate and ethanol concentrations were determined with a FAM-PAK H.P.L.C. column (600E Waters model) on line (Venturin *et al.*, 1995). The lower detection level of H.P.L.C. was 0.1 $g.l^{-1}$

Enzyme assays: Preparation of cell free extracts and enzyme assays of pyruvate decarboxylase (EC 4.1.1.1.), alcohol dehydrogenase (EC 1.1.1.1), pyruvate dehydrogenase (EC 1.2.4.1), $NADP^+$-dependent acetaldehyde dehydrogenase (EC 1.2.1.5 and 1.2.1.4) and acetyl-CoA synthetase (EC 6.2.1.1) have been performed as described by Venturin *et al.* (1995).
Reaction velocities were proportional to the amount of enzyme added. Activities are in $U.(mg\ protein)^{-1}$, in which 1U is defined as the conversion of 1 µmol substrate. min^{-1} at 25°C. The protein content of the cell extracts was measured by the method of Biuret (Stickland, 1951) with bovine serum albumine (Sigma) as a standard.

Presentation of data: Several experimental approaches can be used to study the effects of oxygen on yeast metabolism. One possibility is to study the effect of dissolved-oxygen tension (Furukawa *et al.*, 1983). However, at limiting oxygen supply rates, the dissolved-oxygen tension falls below 2-1 % air saturation and becomes difficult to measure accurately.
An alternative approach is to study the effect of oxygen feed rate (expressed in $mmol.l^{-1}.h^{-1}$) on growth and metabolism of *H. uvarum* K_5 as Weusthuis *et al.* (1994).

Effect of oxygen feed rate on growth of *H. uvarum* K_5

The effect of oxygen on the physiology of *H. uvarum* K_5 was studied by varying the oxygen feed rate to glucose limited chemostat cultures grown at a dilution rate of 0.26 h^{-1}.

Progressive variation of oxygen feed rate

At oxygen feed rates above 80 mmol.l^{-1}.h^{-1} (pO_2 > 2 %), growth of *H. uvarum* K_5 was not oxygen-limited (Figs. 1A and 1B). Glucose was oxidized, as was evident from the absence of ethanol in the culture supernatants and a respiratory quotient (RQ = qCO_2 / qO_2) of near 1. The qO_2 of these oxygen sufficient, glucose limited chemostat cultures was 11 mmol.g^{-1}.h^{-1}. In parallel, physiological parameters were constant and biomass yield was maximum (0.38).

When qO_2 was lowered by decreasing the oxygen feed rate below 80 mmol.l^{-1}.h^{-1}, alcoholic fermentation set in (Fig. 1A). This coincided with a decrease of the biomass yield and with an increase of the qS (Fig. 1B). RQ was higher than 1, metabolism was respiro-fermentative. Ethanol was produced (up to 3.7 g.l^{-1}), but few acetate (0.1 g.l^{-1}).

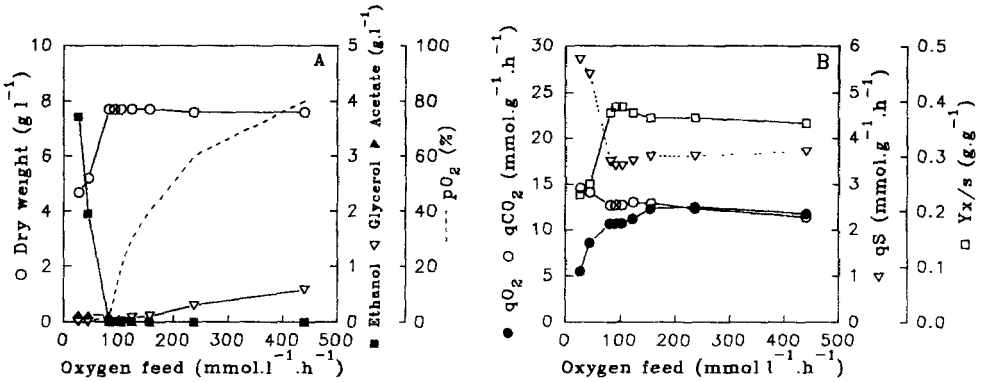

Figure 1. Influence of oxygen feed rate on metabolite and dry weight production **(A)** or on specific rates of oxygen uptake (qO_2), carbon dioxide production (qCO_2), cell yield (Yx/s) and specific rate of glucose consumption (qS) **(B)** of *H. uvarum* K_5 grown in chemostat cultures (D = 0.26 h^{-1}).

The standard deviation in qO_2 and qCO_2 are ≤ 1 % and ≤ 1.5 % (means of five measurements at steady-state). The standard errors in glycerol, acetate and ethanol are ≤ 3 %, ≤ 2.5 % and ≤ 4 % (means of five measurements at steady-state).

Shift of oxygen feed rate

Upon transition from oxygen excess to oxygen limitation, ethanol was excreted immediately (1.83 g.l^{-1} after 2 h.) and little acetate was produced (0.24 g.l^{-1} after 2 h.) (Table 1). After 24 h., biomass yield decreased by 1.8 times and glucose consumption rate increased by 1.7 times. RQ increased to 1.67. These results are in agreement with those obtained in the previous experiment.

Table 1. Product formation, biomass yield (Yx/s), specific rate of glucose consumption (qS) and R.Q. of *H. uvarum* K_5 upon transition from oxygen excess to oxygen limitation in chemostat cultures ($D = 0.26\ h^{-1}$).

Time h	Glycerol $g.l^{-1}$	Acetate $g.l^{-1}$	Ethanol $g.l^{-1}$	Yx/s $g.g^{-1}$	qS $mmol.g^{-1}.h^{-1}$	R.Q.
0	1.40	0.08	0.18	0.42	3.47	1.07
2	1.12	0.24	1.83	0.38	3.76	1.64
4	0.91	0.01	2.88	0.35	4.11	1.73
6	0.80	0	3.25	0.32	4.45	1.62
24	0.80	0	3.16	0.24	5.90	1.67

The standard deviations in glycerol, acetate and ethanol are ≤ 3 %, ≤ 2.5 % and ≤ 4 % respectively.

To investigate wether the observed variations in metabolic fluxes were correlated with an adjustement of enzyme activities, several pyruvate bypass enzymes were analysed in vitro.

Effect of oxygen feed rate on enzyme levels of pyruvate metabolism

Progressive variation of oxygen feed rate

Two responses were observed as a function of oxygen feed rate (Fig. 2). Above oxygen feed rate of 80 $mmol.l^{-1}.h^{-1}$ (pO_2 > 2 %), enzyme activities were constant. Below this value, the levels of fermentative enzymes (alcool dehydrogenase and pyruvate decarboxylase) increased in parallel to ethanol formation. In contrast, the levels of oxydative enzymes (pyruvate dehydrogenase, acetaldehyde dehydrogenase and acetyl-CoA synthetase) strongly decreased.

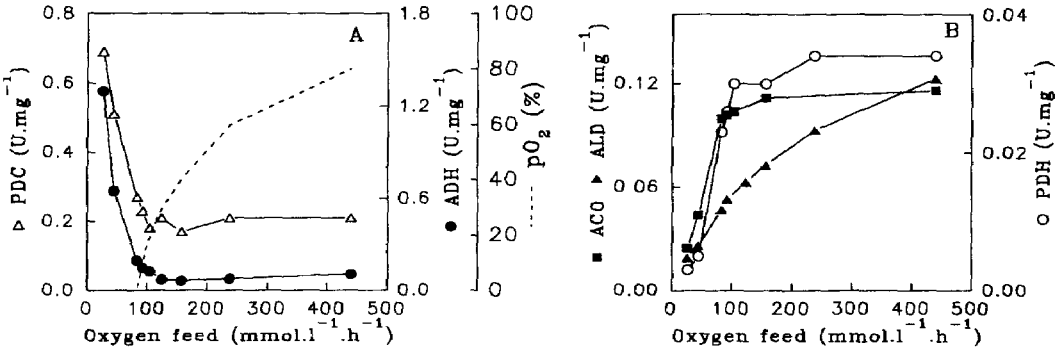

Figure 2. Relation between oxygen feed rate and specific activities (U.mg $protein^{-1}$) of the following enzymes in glucose limited chemostat cultures of *H. uvarum* K_5 at $D = 0.26\ h^{-1}$. **(A)** Alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC). **(B)** $NADP^+$-dependent acetaldehyde dehydrogenase (ALD), acetyl CoA synthetase (ACO) and pyruvate dehydrogenase (PDH).

Data presented here are the means of, at least, triplicate determinations.

Shift of oxygen feed rate

In transient state, an increase in fermentative enzyme activities (alcool dehydrogenase and pyruvate decarboxylase) was immediately observed (Table 2).

In contrast, pyruvate dehydrogenase and $NADP^+$-dependent acetaldehyde dehydrogenase activities strongly decreased. Acetyl-CoA synthetase activity did not vary (0.040 $U.mg^{-1}$).

Table 2. Enzyme activities ($U.mg^{-1}$) under steady-state conditions and after an oxygen limitation of *H. uvarum* K_5 chemostat culture at $D = 0.26\ h^{-1}$.

Time (h)	PDC	ADH	ALD	ACO	PDH
0	0.21	0.10	0.014	0.030	0.017
2	0.60	0.35	0	0.044	0
4	0.73	1.10	0	0.045	0
6	0.72	0.70	0	0.037	0
24	0.71	0.70	0	0.036	0

Enzyme data presented here are means of, at least, triplicate determinations.

These results clearly show that the levels of these enzymes, determined in the transient state after an oxygen limitation vary in the same way as those obtained under steady-state conditions at different oxygen feed rates.

Discussion

In Crabtree-negative yeasts and in Crabtree-positive yeasts grown under glucose limitation, oxygen is a key parameter determining the rate of alcoholic fermentation (van Dijken *et al.*, 1993). The glucose metabolism of *H. uvarum* K_5 enables classification of the strain as a Crabtree-negative yeast (Venturin *et al.*, 1995).

In this strain grown at $D = 0.26\ h^{-1}$, glucose was oxidized up to a dissolved oxygen tension near of zero, corresponding to an oxygen feed rate of 80 $mmol.l^{-1}.h^{-1}$. Below this value, alcoholic fermentation occured and biomass yield decreased from 0.38 to 0.23. The levels of fermentative enzymes (pyruvate decarboxylase and alcohol dehydrogenase) considerably increased but those of respiratory enzymes (pyruvate dehydrogenase, acetaldehyde dehydrogenase and acetyl-CoA synthetase) decreased.

The behaviour of PDC activities in Crabtree-positive and negative yeasts with decreasing oxygen feed rates is different. In the Crabtree-positive yeast *S. cerevisiae*, high levels of pyruvate decarboxylase (0.67 $U.mg^{-1}$) are present even under aerobic, glucose-limited conditions (Pronk *et al.*, 1994). These activities increase only twofold under respiro-fermentative conditions (Weuthuis *et al.*, 1994). In contrast, only low PDC activities could be detected in aerobic glucose limited cultures of Crabtree-negative yeasts like *H. uvarum* K_5 (0.20 $U.mg^{-1}$) or *C. utilis* (0.30 $U.mg^{-1}$) (Weuthuis *et al.*, 1994). These activities increased sharply when *H. uvarum* K_5 (x 3.5) or *C. utilis* (x 7) were grown under oxygen limitation. In *H. uvarum* K_5 or *C. utilis*, alcoholic fermentation occured only if oxygen was the limitant factor.

These data suggest that regulation of glucose metabolism at the level of pyruvate in the Crabtree-negative yeast *H. uvarum* K_5, as in *C. utilis*, may be controled by oxygen level (Weusthuis *et al.*, 1994).

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