0273-2289/92/3435—0369$02.00

The Effect of Oxygenation on Glucose Fermentation with *Pichia stipitis*

Scientific Note

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Index Entries: Xylose fermentation; *Pichia stipitis;* oxygenation; glucose fermentation; redox balance.

INTRODUCTION

Pentose fermentation is a problem for the biotechnological industry concerned with the efficient utilization of plant biomass for ethanol production. Lignocellulosic biomass consists of hemicellulose, cellulose, and lignin. The main component in hemicellulose is xylose, and in cellulose, it is glucose. These sugars can be fermented to fuel ethanol. *Pichia stipitis* is one of the better xylose-fermenting yeasts (*1–3*), but it requires carefully controlled oxygenation for efficient ethanol production (*4,5*). Oxygen has also been shown to be important for ethanol production from glucose (*4,6*). Therefore, the fermentation of lignocellulosic hydrolysates—containing both hexoses and pentoses—with *P. stipitis* requires well-controlled fermentations for efficient ethanol production.

The role of oxygen for efficient ethanol formation during pentose fermentations has given rise to several hypotheses about the xylose metabolism:

1. Oxygen is necessary to maintain the redox balance in the first two steps of the xylose metabolism (*7–9*);

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2. Oxygen is necessary for the transport of xylose (*10,11*);
3. Oxygen is necessary for growth; and
4. Oxygen is necessary for unimpaired mitochondrial function (*12*).

With the aim of further elucidating the role of oxygen in maintaining the redox balance in the first two steps during the xylose metabolism, we investigated the fermentation of glucose in continuous culture with either oxygen limitation and an excess of glucose or glucose limitation and an excess of oxygen. At each steady state, product formation, intracellular enzyme activities (pyruvate decarboxylase [PDC], malate dehydrogenase [MDH]), and concentrations of intracellular intermediary metabolites (fructose 1.6-diphosphate [FDP], pyruvate [Pyr], and malate [Mal]) were measured.

MATERIALS AND METHODS

Strain

Pichia stipitis CBS 6054 was maintained as described earlier (*5*).

Cultivation Conditions

Medium

Medium consisted of glucose (50 g/L), 6.7 g yeast nitrogen base/L (Difco Laboratories, Detroit, MI), 0.81 g K_2HPO_4/L, and 12.90 g KH_2PO_4/L. In the carbon-limited culture, 20 g/L glucose were used.

Fermentor

The fermentor consisted of a 1.5-L glass vessel (Applicon Dependable Instruments, Netherlands) with an EFC 24 control system (Electrolux Fermentation, Getinge, Sweden).

Conditions

The temperature was set to 30°C; pH was controlled to 5.5. Agitation varied between 400–1000 rpm, and the air flow from 0–0.9 L/min to obtain an oxygen consumption rate between 0–64 mmol/L h. Dissolved oxygen was measured with a polarographic electrode (Ingold Messtechnik AC, Switzerland). Oxygen (gas) was measured by an Oxygenos-1 (Leybold-Heraeus GMBH Köln, Germany). Carbon dioxide was measured by a Binos infrared gas analyzer (Leybold-Heraeus).

Batch Fermentation

Cells grown under oxygen-limited conditions were inoculated into a nitrogen-flushed fermentor, and the culture was then sparged with 1.0 L/min nitrogen.

Analytical Methods

Glucose, ethanol, acetic acid, and glycerol were analyzed by HPLC (*13*). Cell dry weights were measured as earlier described (*5*).

Intermediary Metabolites

Perchloric acid extracts for the determination of intermediary metabolite concentrations were prepared (*5*). They were assayed enzymatically and detected spectrofluorometrically. FDP and Pyr were determined according to Lowry and Passonneau (*14*). MAL was determined according to Williamson and Corkey (*15*). Each metabolite concentration was determined as the mean of six independently filtered, frozen, and extracted samples.

Intracellular Enzyme Activities

Cell extracts were prepared by breaking the cells by freeze pressing (two passes) in an X-Press. Cell debris was removed by centrifugation, and the supernatant was frozen and stored at −80°C until analyzed (*5*). PDC, MDH, and protein were measured as described earlier (*15–17*). The specific activity of the enzymes was expressed as mmol of converted substrate/g of protein min.

RESULTS AND DISCUSSION

Product Formation and Substrate Consumption

In a fully aerated carbon-limited culture, no ethanol was produced (Fig. 1a). Under oxygen limitation with glucose in excess, a maximum specific productivity of 0.38 g/g h and a maximum ethanol yield of 0.33 g/g consumed glucose were achieved at an oxygen consumption rate below 8 mmol/L h (Fig. 1a). In order to further expand the oxygen limitation without causing wash-out, an anaerobic batch fermentation was performed. The specific ethanol productivity decreased to 0.025 g/g h. In this culture, it was measured when there was a linear relation between both glucose consumption and ethanol production vs time. In another strain of *P. stipitis* (CBS 5773), a maximum specific ethanol productivity of 0.35 g/g h was obtained at an oxygen consumption rate below 5 mmol/L h (*6*). In comparison, the highest specific ethanol productivity (0.20 g/g h) from xylose has been reached with *P. stipitis* CBS 6054 (*5*), whereas *P. stipitis* CBS 5773 reached 0.13 g/g h (*13*). These results obtained in continuous cultures confirm that the specific ethanol productivity is higher with glucose than with xylose for *P. stipitis*, which has previously been reported for batch cultures (*4*).

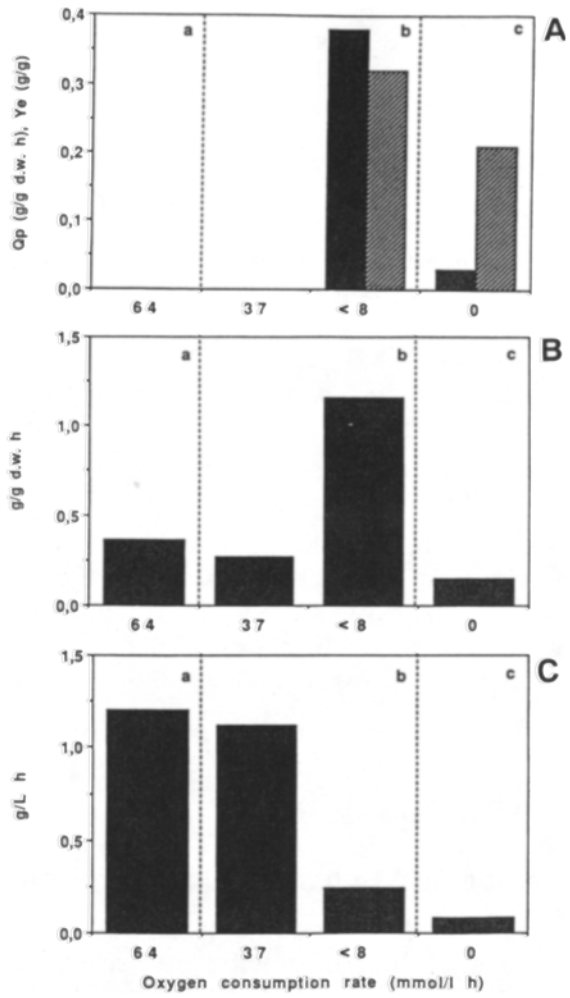

Fig. 1. Specific ethanol productivity (Qp), ethanol yield (Ye) (A), specific glucose consumption rate (B), and cell growth (C) vs oxyen consumption rate. Legends within the figures: **a** carbon-limited culture, **b** oxygen-limited culture, **c** anaerobic batch culture.

The specific glucose consumption rate reached its highest value at maximum specific ethanol productivity. The consumption rate was very low in the anaerobic culture (Fig. 1b). The cell growth decreased with decreased oxygenation (Fig. 1c). However, unlike the case of xylose fermentation, *P. stipitis* grew under all culture conditions, even if the growth rate was very low anaerobically (Fig. 1c). The fact that *P. stipitis* does not grow anaerobically on xylose, but produces small amounts of ethanol (*5*) leaves the question of whether ethanol formation is growth related unanswered.

Apart from cell mass, ethanol, and carbon dioxide, small amounts of acetic acid (0–0.06 g/g h) were produced, but no glycerol (data not

Table 1
Intracellular Intermediary Metabolite Concentration and Enzyme Activity vs Oxygen Consumption Rate

Oxygen uptake, mmol/L h	Glycolysis			TCA cycle	
	FPD[1]	Pyruvate[1]	PDC[2]	Malate[1]	MDH[2]
64	0.05±0.006[3] (0.74)[4]	0.13±0.01 (1.96)	0.07±0.002	0.30±0.02 (4.62)	15±0.5
37	0.18±0.01 (2.60)	0.29±0.01 (4.08)	0.19±0.01	0.37±0.02 (5.19)	18±1
<8	0.40±0.04 (7.51)	0.24±0.001 (4.52)	0.33±0.002	0.64±0.02 (12.1)	12±1

[1]μmol/mg dw/mmol glucose/L h.
[2]mmol/g prot. min.
[3]Standard deviation.
[4]Numbers in brackets shows the metabolite conc. before standardization (μmol/mg dw).

shown). The carbon mass balance was almost closed (85–102%) (data not shown), indicating that no other byproducts were obtained. The cell dry weight was converted to moles carbon by using the elementary composition formula reported for *Saccharomyces cerevisiae*, $CH_{1.83}O_{0.56}N_{0.17}$ (*18*).

Activity in Glycolysis

In order to discriminate the effect of oxygenation from the effect of the glucose consumption, as well as to enable a comparison with other carbon sources (*19,20*), the intermediary metabolite concentrations shown in Table 1 were obtained by dividing by the respective overall glucose consumption rates, expressing them as μmol/g dry wt/mmol/L h. The intracellular concentrations of FDP increased when oxygenation decreased (Table 1), which is in agreement with the results obtained in xylose-fermenting cells of *P. stipitis* (*5*). The accumulation of FDP might indicate that the metabolism was slowed down when oxygenation was decreased. The concentrations of Pyr reached a maximum in the middle of the investigated oxygenation range (Table 1), whereas in xylose-fermenting cells, the concentration decreased with decreasing oxygenation (*5*). PDC activity increased in both glucose (Table 1) and xylose-fermenting cells when oxygenation decreased (*5*).

Activity in TCA-Cycle

Mal increased (Table 1) when oxygenation decreased, and this has also been found for xylose-fermenting *P. stipitis* (*5*). In xylose-fermenting *P. stipitis*, the MDH activity decreased with decreasing oxygenation, whereas this was not equally clear in glucose-fermenting cells (Table 1).

The fact that decreasing oxygenation influenced intracellular metabolites and enzymes similarly in glucose-fermenting and xylose-fermenting cells of *P. stipitis* suggests that oxygen is not necessary to maintain the redox balance. The supposed redox imbalance is derived from the first two steps in the xylose metabolism, where xylose is reduced to xylitol via a xylose reductase and thereafter is oxidized to xylulose with a xylitol dehydrogenase (*7–9*). These steps are not working in glucose-fermenting cells (*21*). This is further supported by the earlier result that oxygenation does not influence the activity of xylose reductase and xylitol dehydrogenase (*5*). Therefore, the prerequisite of oxygenation for efficient ethanol formation from both glucose and xylose in *P. stipitis* must be ascribed to sugar transport, growth, or an unimpaired mitochondrial function.

ACKNOWLEDGMENTS

This study was supported by the Swedish National Energy Administration. Sven Törnquist is thanked for excellent technical assistance and Christer Holmgren for linguistic advice.

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