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conference paper

Induction of Oxidofermentative Ethanol Formation in Recombinant Cells of *Saccharomyces cerevisiae* Yeast

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Summary

So-called Crabtree-positive yeasts, such as Saccharomyces cerevisiae, ferment glucose to ethanol even under fully aerobic conditions, when the sugar is present in excess. This metabolic behaviour leads to a decrease of the biomass yield and it is therefore disadvantageous for recombinant protein production. Using a strain of Sacch. cerevisiae expressing garlic alliin lyase, we found that the specific glucose uptake rate is an important parameter for the induction of oxidofermentative metabolism, with a threshold value for the onset of ethanol formation of 0.2 g g^{-1} h^{-1} (glucose per dry biomass and hour). It can be concluded that an optimal production process has to run under strict control of glucose feeding in order not to exceed this critical value of specific glucose uptake rate.

Key words: Saccharomyces cerevisiae, Crabtree effect, chemostat culture, recombinant alliin lyase

Introduction

Saccharomyces cerevisiae belongs to the large group of facultatively fermentative yeasts which can ferment sugars with ethanol being the main by-product. In so-called Crabtree-positive yeasts (like *Sacch. cerevisiae*), alcoholic fermentation occurs under strictly aerobic conditions in the presence of excess sugar. Crabtree-negative yeasts on the other hand (like *Pichia pastoris*) do not ferment sugar under fully aerobic conditions.

The onset of aerobic fermentation is most often correlated to the specific growth rate, or the dilution rate in chemostat cultures, respectively (*1*). In batch cultures, the sugar concentration in the medium is often mentioned as the cause for aerobic fermentation (*2*).

Due to the low binding affinity of pyruvate decarboxylase (leading to ethanol formation) compared to pyruvate dehydrogenase (leading to the respirative pathway), the internal pyruvate concentration plays a major role in the modulation of the Crabtree effect (*3*). Data from the literature do not allow the conclusion that a bottleneck exists in the respirative catabolism of pyruvate, but it has been shown that excessive glucose uptake leads to an increase of pyruvate and finally to ethanol formation. Van Dijken *et al.* (*3*) mention a substantial difference between Crabtree-positive and-negative yeasts: Crabtree-negative yeasts contain energy-consuming glucose carriers, whereas Crabtree-positive yeasts transport glucose *via* facilitated diffusion.

Aerobic ethanol formation can lead to a dramatic reduction of biomass yield and hence of recombinant product yield. Additionally, the stability of a production process cannot be guaranteed as long as the parameters leading to ethanol formation are not clarified.

Therefore we have investigated which parameters determine the onset of aerobic ethanol formation in two recombinant strains of *Sacch. cerevisiae*. We have run chemostat cultures at different dilution rates and with different glucose concentrations in the feed.

All experiments were performed with recombinant yeast strains producing garlic alliin lyase (*4*).

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Materials and Methods

Yeast strains

AH22 (a, leu2-3, leu2-112, his4-519, can1) was used in experiments 1–4.

M3585 (a, ura3, leu2, his3, pep4, prb1) was used in experiment 5.

The main difference between the two strains is the deficiency of M3585 for two proteases. Two strains were utilised in order to test the broader validity of the results.

Expression vector

pPTAllii carries the gene for garlic alliin lyase, cloned into pPT2bL2 (5) so that it is controlled by the alcohol dehydrogenase 1 (ADH1) promoter. ADH1 of *Sacch. cerevisiae* catalyses the regeneration of NAD^+ from NADH with the formation of ethanol from acetaldehyde. The enzyme is induced by fermentable carbon sources like glucose (6). The plasmid pPTAllii is maintained in the host by a 2 μm origin.

Growth media

Four chemostat experiments were carried out in the synthetic medium SD^-. In experiment 1 we used Yeast Nitrogen Base (YNB) without amino acids (6.7 g L^{-1}); glucose (4.55–20 g L^{-1}); uracil (20 mg L^{-1}); histidine (15 mg L^{-1}).

In experiment 2, the glucose concentration in the feed (S_0) was 10 g L^{-1}, and all other components were kept as above. In experiments 3 and 4, the glucose concentrations in the feed was 16 g L^{-1} and 25 g L^{-1} respectively, and the concentrations of all components were elevated in the same relation to glucose.

Experiment 5 was conducted in the semi-complex medium SK:

$(NH_4)_2SO_4$ (2.5 g L^{-1}); KH_2PO_4 (0.5 g L^{-1}); yeast extract (1.0 g L^{-1}); $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}); $CaCl_2 \cdot 2H_2O$ (0.02 g L^{-1}); $FeCl_3 \cdot 6H_2O$ (4.0 mg L^{-1}); $ZnSO_4 \cdot 7H_2O$ (2.0 mg L^{-1}); glucose (10.0 g L^{-1}); uracil (20.0 mg L^{-1}); histidine 20.0 mg L^{-1})

Chemostat experiments

All experiments were carried out using a CMF Mini Fermenter with 1.2 L working volume. The setpoints during fermentation were *pH* = 6.0 ± 0.1; t = (30.0 ± 0.1) °C; DO = (50.0 ± 0.5) % air saturation. Agitation (400–900 r.p.m) was cascade controlled, dependent on DO value. All setpoints were PI controlled.

The fermentation was controlled by a CBS10 Control System (microprocessor-based digital control system).

Experiment 1 was run at constant dilution rate D = 0.04 h^{-1} with raising glucose concentration in the feed (4.55g L^{-1}, 9.09 g L^{-1}, 14.54 g L^{-1} and 20.0 g L^{-1}), and in experiments 2–4 dilution rate was increased from around 0.02 h^{-1} up to 0.345 h^{-1} with constant glucose concentration in the feed (10 g L^{-1}, 16 g L^{-1} and 20 g L^{-1}, respectively).

In Experiment 5 the dilution rate was increased from 0.05 h^{-1} to 0.2 h^{-1}.

Analysis of the culture supernatants

Ethanol and D-glucose were quantified with the respective Boehringer Mannheim (D) test combinations.

Yeast dry mass (X) was quantified after centrifugation, washing and drying of samples at 105 °C for 24 hours.

Results and Discussion

When we related ethanol formation to glucose concentration or growth rate (dilution rate), no clear correlation could be observed, as ethanol was produced even at very low growth rates (μ = 0.04 h^{-1}) in some cases. However, the specific rate of ethanol formation per biomass (ρ) and the specific rate of glucose uptake per biomass (ν) showed a strong linear correlation (Fig. 1). Below a threshold value which is estimated around ν = 0.2 g g^{-1} h^{-1} (glucose per biomass and hour), aerobic fermentation did not occur. The linear trend above this threshold (Fig. 1) had a correlation coefficient r = 0.994.

From these data, we concluded the following relation between ρ and ν:

For $\nu < 0.2$ g g^{-1} h^{-1} $\quad \rho = 0$ /1/

For $\nu \geq 0.2$ g g^{-1} h^{-1} $\quad \rho = 0.35\,\nu - 0.070$ /2/

Apparently, in a chemostat culture in a steady state, glucose uptake depends on the dilution rate (D), the glucose concentration in the feed (S_0) and the biomass yield (Y_{SX}) which determines the actual amount of biomass produced in the chemostat, and hence the biomass concentration in the fermenter.

From

$$\nu = \frac{(S_0 - S) \cdot D}{X} \qquad /3/$$

and

$$X = (S_0 - S) \cdot Y_{SX} \qquad /4/$$

follows

$$\nu = \frac{D}{Y_{SX}} \qquad /5/$$

That means that in a steady-state Y_{SX} determines ν at constant D. A decrease of Y_{SX} for some reason like in-

Table 1. Steady-state values from a chemostat culture of *Sacch. cerevisiae* M3585 with SK medium (S_0 = 10 g L^{-1}) at different dilution rates (Experiment 5).

D	γ(X)	γ(glucose)	γ(ethanol)
h^{-1}	g L^{-1}	g L^{-1}	g L^{-1}
0.05	2.36	0.037	0.918
0.10	1.71	0.0185	2.915
0.15	1.31	0.0185	3.584
0.20	1.34	0.115	3.939

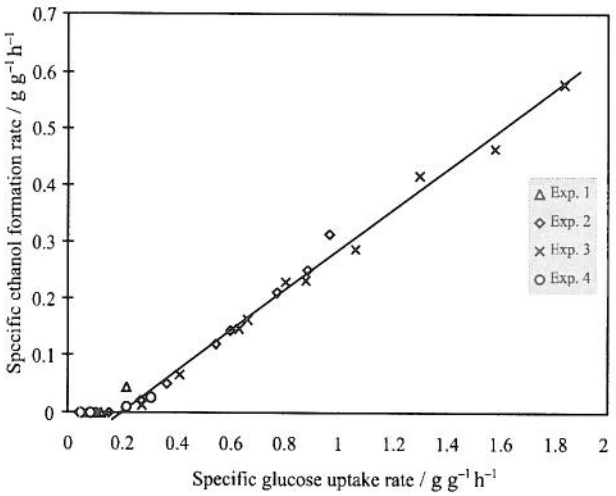

Fig 1. Specific ethanol formation rate ρ versus specific glucose uptake rate ν in a chemostat culture of *Sacch. cerevisiae* AH22 with SD^- medium at different dilution rates with different substrate concentrations in the feed.
Exp. 1: constant dilution rate (0.04 h^{-1}), increasing glucose concentrations in the feed
Exp. 2: increasing dilution rates, constant glucose concentration in the feed (10 g L^{-1})
Exp. 3: increasing dilution rates, constant glucose concentration in the feed (16 g L^{-1})
Exp. 4: increasing dilution rates, constant glucose concentration in the feed (25 g L^{-1})

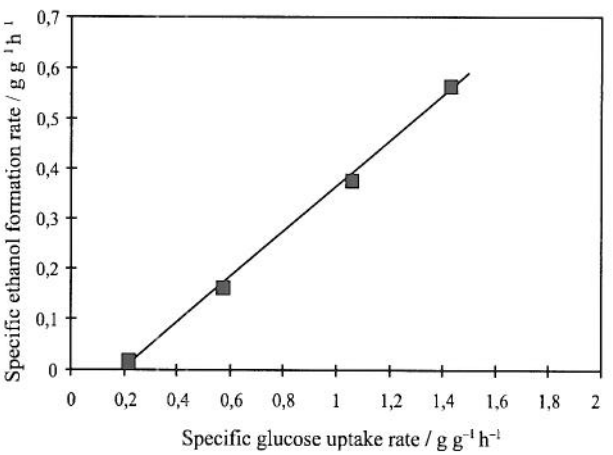

Fig. 2. Specific ethanol formation rate ρ versus specific glucose uptake rate ν in a chemostat culture of *Sacch. cerevisiae* M3585 with SK medium (S_0 =10 g L^{-1}) at different dilution rates (Experiment 5)

complete mixing, local anaerobic conditions etc., leads to a decrease of X. If the glucose uptake into the cell would be strictly limited (as with Crabtree-negative yeasts), this would lead to an accumulation of residual substrate. *Sacch. cerevisiae*, on the contrary, will take up the then higher supply of substrate, so that ν can exceed the critical threshold value, leading to fermentation and a further decrease of Y_{SX}. This may result in an alternative steady state with low Y_{SX} and ethanol formation even at a subcritical dilution rate.

Cultivation 5 demonstrates such a development. When the feed was started at D = 0.05 h^{-1}, X was 4 fold lower than the theoretical 5 g L^{-1}. As shown in Table 1, the residual glucose in the steady state at D = 0.05 h^{-1} was almost 0, although the biomass concentration reached only 2.36 g L^{-1}, and approx. 1 g L^{-1} ethanol was accumulated. As ν exceeded 0.2 g g^{-1} h^{-1} from the beginning even at the low dilution rate of 0.05 h^{-1}, we concluded that aerobic ethanol formation, resulting in a decreased biomass yield, prevented the culture from reaching a steady state without ethanol formation. The ethanol formation rate (Fig. 2) again followed the same relation to glucose uptake rate as stated before. This demonstrates that Equations 1 and 2 are valid for different strains and different growth media.

The analysis of batch and fed batch cultures also verified the same linear relation between ρ and ν (data not shown). Generally we concluded that an optimal production process has to be run under strict control of glucose feeding in order not to exceed the critical value of glucose uptake rate.

It should by noted that under these aspects, the use of glucose-induced promoters (like the alcohol dehydrogenase 1 or the phosphoglycerate kinase promoter) for recombinant protein expression is not feasible, as the conditions suitable for avoiding aerobic fermentation do not allow for full induction of these promoters.

Acknowledgements

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List of Symbols

S_0 / g L^{-1}	glucose concentration in the feed
S / g L^{-1}	glucose concentration in the culture supernatant
X / g L^{-1}	dry biomass concentration in the culture
Y_{sx}/ g g^{-1}	yield of biomass per glucose
D / h^{-1}	dilution rate
ν / g g^{-1} h^{-1}	specific glucose uptake rate
ρ / g g^{-1} h^{-1}	specific ethanol formation rate
μ / h^{-1}	growth rate

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Indukcija oksidofermentativne tvorbe etanola u stanicama rekombinantnog kvasca *Saccharomyces cerevisiae*

Sažetak

»Crabtree« pozitivni kvasci, kao što je Saccharomyces cerevisiae, fermentiraju glukozu u etanol, čak i u potpuno aerobnim uvjetima ako je dovoljno velika koncentracija šećera. Taj oblik metabolizma uzrokuje smanjenje prinosa biomase i zbog toga je nepovoljan za proizvodnju proteina postupkom rekombinantne DNA. Na primjeru Sacch. cerevisiae, koji sadržava gen za aliin liazu iz češnjaka, utvrđeno je da je specifična brzina potrošnje glukoze važan parametar indukcije oksidofermentativnog metabolizma, a granična je vrijednost pri kojoj započinje nastajanje etanola 0,2 g g^{-1} h^{-1}. Može se zaključiti da se za optimalnu proizvodnju etanola treba strogo kontrolirati dotok glukoze kako se ne bi prekoračila kritična vrijednost specifične brzine potrošnje glukoze.