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Biosynthesis regulation of the β-glucosidase produced by a yeast strain transformed by genetic engineering

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Abstract. The biosynthesis of the β-glucosidase enzyme was studied in a transformed yeast obtained by cloning in *Saccharomyces cerevisiae* the structural gene coding for β-glucosidase in *Kluyveromyces fragilis*. The enzyme biosynthesis was found to be non-adaptative, and repressed by glucose. These features are similar to those observed in *K. fragilis*. β-Glucosidase activity in the transformed yeast was much higher than in *K. fragilis*. We attempted to ferment cellobiose with the transformed yeast: practically no cellobiose was consumed, growth and ethanol production were negligible. Warburg experiments showed that cellobiose fermentation did not occur when the respiratory chain was not functioning.

Key words: β-Glucosidase – Yeast – Genetic engineering – Biosynthesis regulation

Ethanol production from lignocellulosic feedstocks can be achieved by several means, one of which is called "Simultaneous saccharification and fermentation": cellulose hydrolysis and fermentation of the soluble sugars are performed by a cellulolytic fungus and a yeast strain capable of fermenting both glucose and cellobiose (Blotkamp et al. 1978; Vause 1983). The cellulase system of cellulolytic fungi generally contains only small amounts of β-glucosidase. This causes cellobiose to accumulate and strongly inhibit β-glucanases (Emert et al. 1974; Sternberg 1976). It is thus necessary to realize the fermentation step with a yeast strain producing large amounts of β-glucosidase. In the past years, we selected yeast strains fermenting glucose and cellobiose anaerobically (Gondé et al. 1982). However, these strains fermented cellobiose slowly, and often incompletely. Moreover, cellobiose fermentation was compromised in the presence of glucose due to catabolic repression phenomena.

One yeast species, *Saccharomyces cerevisiae* Hansen, ferments glucose rapidly and with good yields, but does not produce β-glucosidase, and is therefore not capable of fermenting cellobiose. In order to overcome this problem, Raynal and Guérineau (1984) have cloned the structural gene coding for β-glucosidase from *Kluyveromyces fragilis* van der Walt into *Saccharomyces cerevisiae*. In the strain thus obtained (called TYKF2), the β-glucosidase gene was expressed at a level much higher than in *K. fragilis*. This paper deals with the study of the regulation of β-glucosidase biosynthesis in strain TYKF2, and the lack of fermentation of cellobiose by this strain.

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Materials and methods

Strains. The strains used were *Kluyveromyces fragilis* Y610 (ATCC 12424) and *Saccharomyces cerevisiae* TYKF2. The latter was obtained by Raynal and Guerineau (1984) by cloning in *S. cerevisiae* the structural gene coding for β-glucosidase biosynthesis in *K. fragilis* strain Y610. They used the cosmid pHCG3, containing a segment of the yeast 2 μ plasmid, the yeast URA3 gene, and the bacterial cosmid pHC79 (Hohn and Collins 1980).

Culture conditions. The basal medium was G medium (Galzy and Slonimski 1957), supplemented with casamino-acids (which improve the growth rate of strain TYKF2), histidine and leucine (TYKF2 is auxotrophic for histidine and leucine). Casamino-acids were supplied at a concentration of 20 $g \cdot l^{-1}$, while histidine and leucine final concentration was 50 $mg \cdot l^{-1}$.

Aerobic cultures were performed at 28° C in Erlenmeyer flasks filled to one-tenth of their volume and shaken. Anaerobic cultures were performed in Erlenmeyer flasks filled to 40% of their volume; oxygen was removed from the medium by bubbling nitrogen gas. Anaerobic culture media were supplemented with Tween 80 (0.8%) and ergosterol ($0.05 g \cdot l^{-1}$).

Cell grinding conditions. Cells were ground according to the method previously described (Leclerc et al. 1984).

Enzyme and protein assay. β-Glucosidase activity was assayed at 30° C and pH 6 in 0.1 M citrate-phosphate buffer according to the method previously described (Blondin et al. 1983), pH 6 was chosen because it was the enzyme's optimum pH, determined in vitro on crude extracts. The activity was determined using p-nitrophenyl-β-D-glucopyranoside as the substrate. One enzyme unit is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per min and per mg of protein (I. U.).

Proteins were assayed according to the method of Lowry et al. (1951).

Manometric techniques. Q_{O_2} and Q_{CO_2} were determined by the Warburg manometric technique according to Umbreit et al. (1964).

Results

Regulation of β-glucosidase biosynthesis in Saccharomyces cerevisiae strain TYKF2. β-Glucosidase activity was assayed

Table 1. β-Glucosidase in *Saccharomyces cerevisiae* TYKF2 and *Kluyveromyces fragilis* Y610 cells following aerobic culture on several substrates. Growth rate of strain TYKF2 on cellobiose: $0.15\ h^{-1}$

Growth substrate	Activity of ground cells (I.U.)	
	K. fragilis Y610	*S. cerevisiae* TYKF2
Cellobiose $5\ g \cdot l^{-1}$	0.025	5.8
Cellobiose $50\ g \cdot l^{-1}$	0.051	6.9
Glucose $5\ g \cdot l^{-1}$	0.006	4.8
Glucose $50\ g \cdot l^{-1}$	0.003	2.5
Glycerol $10\ g \cdot l^{-1}$	0.047	6.0
Methyl-β-D-glucose $5\ g \cdot l^{-1}$	0.055	9.0

on ground cells following aerobic growth with different carbon sources at the $5\ g \cdot l^{-1}$ and $50\ g \cdot l^{-1}$ concentrations (Table 1). Cells were harvested at the end of log phase. We had previously checked that β-glucosidase activity was exclusively intracellular and was constant over growth. The enzyme was synthesized in similar amounts when the growth substrate was cellobiose or glycerol. However, growth on glucose resulted in lower β-glucosidase levels. This effect was not very marked, but became stronger with increasing glucose concentration in the medium.

A similar study was realized with *K. fragilis* strain Y610. It appeared that β-glucosidase biosynthesis was the same following culture on cellobiose or glycerol. The "glucose effect" was more pronounced than of strain TYKF2. However, the activity level in the Y610 strain was much lower than in TYKF2, following growth on cellobiose (Table 1).

Fermentation tests on glucose and cellobiose. We tried to realize with strain TYKF2 the anaerobic fermentation of glucose and cellobiose. Glucose was totally consumed in 24 h at the $60\ g \cdot l^{-1}$ concentration. When supplied at the same concentration, only $4\ g \cdot l^{-1}$ cellobiose were consumed, and the number of cells in the medium was only doubled (attaining $60 \cdot 10^6$ cells $\cdot$ ml^{-1}), following 3 days incubation; alcohol production was negligible. When a glucose-cellobiose mixed fermentation was attempted, glucose was fermented while cellobiose was not.

Influence of oxygen on β-glucosidase biosynthesis. In order to determine whether the lack of cellobiose fermentation was due to the absence of enzyme biosynthesis in anaerobic conditions, we ground cells following anaerobic culture on glucose ($5\ g \cdot l^{-1}$). β-Glucosidase activity was assayed: it was similar to the one determined after aerobic culture on glucose ($5\ g \cdot l^{-1}$), namely 5 I. U. Oxygen was thus not crucial for β-glucosidase biosynthesis by strain TYKF2.

Influence of the respiratory chain on cellobiose fermentation. Following growth on cellobiose, TYKF2 cells were washed twice with phosphate buffer (50 mM; pH 4.5), then put into Warburg vessels. Respiratory and fermentation coefficients were determined with cellobiose as the carbon source. No nitrogen source was added, in order to stay in non-growing conditions. Anaerobic conditions were achieved by flushing nitrogen gas into Warburg vessels during 5 min, prior to measurement beginning.

In anaerobic conditions, no CO_2 was produced from cellobiose. When glucose was added to the medium, CO_2 production began, indicating normal fermentation of this sugar. In aerobic conditions, cellobiose was metabolized by the oxydative pathway, but also slowly fermented. When conditions were shifted to anaerobiosis, or when potassium cyanide was added to the medium, CO_2 production stopped. A subsequent addition of glucose led CO_2 production to start again. Thus, cellobiose fermentation by TYKF2 strain is not possible when the respiratory chain does not work.

Discussion

The regulation of β-glucosidase biosynthesis was found to be similar in *K. fragilis* strain Y610 and in *S. cerevisiae* strain TYKF2: in both organisms cellobiose did not act as inducer, but growth in the presence of glucose led to decreased enzyme levels.

Although the TYKF2 strain did grow aerobically on cellobiose as the sole carbon source, it failed in fermenting this sugar in anaerobic conditions. The non-fermentation of a disaccharide in anaerobic conditions by a yeast strain capable of using it aerobically has been previously described and called Kluyver effect (Barnett 1981). This feature was explained by a failure of disaccharide transport into yeast in anaerobiosis (Sims and Barnett 1978), or by a negative regulation exerted on the pathway from pyruvate to ethanol (Barnett and Sims 1982). We demonstrated that in strain TYKF2 β-glucosidase was synthesized in anaerobiosis as well as in aerobiosis. Thus non-fermentation of cellobiose by this strain was not due to the absence of enzyme biosynthesis. Warburg experiments showed that cellobiose fermentation was not possible when respiration was not functioning, while glucose was fermented in these conditions. It seems thus probable that cellobiose transport requires an energy supply involving the respiratory chain. These findings are consistent with others on a strain of *K. lactis* (Entian and Barnett 1983).

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