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Biochemical Studies on the Production of Acetic Acid by the Yeast *Dekkera anomala*

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Summary

Cells of *Dekkera anomala* IGC 5153 grown in concentrations of glucose higher than 2 % (w/v) were able to produce high amounts of acetic acid, while in lower sugar concentrations the presence of the acid was not detected. Ethanol was detected when the glucose concentration in the culture medium was above 2 % and in all growth conditions production of glycerol was not found. The fermentative metabolism appeared to be the main pathway involved in glucose catabolism. The enzyme acetyl-CoA synthetase was strongly repressed by glucose and the high acetic acid concentrations found in the culture medium resulted probably from the insufficient activity of the acetyl-CoA synthetase required for the complete conversion of acetate to acetyl-CoA.

Key words: acetic acid production, acetyl-CoA synthetase, *Dekkera anomala*, yeast

Introduction

The species of the genus *Dekkera/Brettanomyces* have been described as spoiler yeasts of grape musts and fermented beverages. In wine industry these yeasts are responsible for important economic losses because of their capacity to consume residual sugars and to produce obnoxious flavour and odours (*1*,*2*). These species are well known by their capacity to produce large amounts of acetic acid from growth on glucose. Particularly, during alcoholic fermentations such high amounts of acetic acid may have negative effects on the growth of the fermentative yeast *Saccharomyces cerevisiae*.

It has been proposed that in *Dekkera/Brettanomyces* the formation of acetic acid is linked to the reaction of acetaldehyde oxidation with concomitant reduction of $NAD(P)^+$. The NAD^+- and $NADP^+$-acetaldehyde dehydrogenase enzymes are involved in this process, and the second one is apparently constitutive at least in *Brettanomyces abstinens* (*3*). The continued conversion of acetaldehyde to acetate, in anaerobic conditions, led to the disturbance of the redox balance (NAD^+/ NADH) with the subsequent stagnation of the glycolytic flux (Custers effect). The absence of glycerol production by species of this genus under the mentioned growth conditions seems to account for their inability to restore the redox balance (*4*).

The aim of this work was to elucidate the metabolic pathways which could be involved in the production of acetic acid by *Dekkera anomala*. In this paper the strain IGC 5153 was used and experimental evidence confirmed that high amounts of acetic acid produced by that species were related to the repression of acetyl-CoA synthetase by high glucose concentrations in the growth medium.

Materials and Methods

Microorganism and growth conditions

Dekkera anomala IGC 5153 was maintained on a medium containing glucose (2 %, w/v), peptone (1 %, w/v), yeast extract (0.5 %, w/v), and agar (2 %, w/v). The cells were grown in 500 mL flasks with 200 mL of liquid

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mineral medium containing vitamins (*5*) supplemented with glucose (from 0.1 to 20 %, w/v), ethanol (0.5 %, v/v), or acetic acid (0.5 %, v/v). All cultivations were carried out at the initial pH value of 5.5 and 26 °C with mechanical shaking (150 rpm). Growth was monitored by measuring the absorbance at 640 nm, $A_{640\ nm}$ (Spectronic 21, Bausch & Lomb) for determination of maximum specific growth rates (μ_{max}).

Preparation of cell extracts and enzyme assays

Yeast cells were harvested in the mid-exponential phase of growth, washed twice with ice-cold distilled water and the cell-free extracts were prepared as described by Perea and Gancedo (*6*). Briefly, 30 mg of cells (wet weight) were mixed with 0.75 g glass beads (0.5 mm diameter) and 0.5 mL 20 mM imidazole buffer (pH=7.0) and vortexed for four periods of 1 min, with 1 min interval on ice between them. After centrifugation at 15000 ×g for 15 min at 4 °C, enzyme activities were measured in the supernatant, using the following described procedures: acetyl-CoA synthetase (EC 6.2.1.1), alcohol dehydrogenase (EC 1.1.1.1) and NAD^+-acetaldehyde dehydrogenase (EC 1.2.1.5) (*7*); malate dehydrogenase (EC 1.1.1.37) (*8*); glucose phosphorylating enzymes (G-Phosp) and fructose phosphorylating enzymes (F-Phosp) (*9*). Assays were carried out in a LS 50 UV/VIS spectrometer (Perkin Elmer) at 26 °C. Protein was determined by the Lowry method (*10*) with bovine serum albumin as the standard.

Estimation of acetic acid, ethanol and glycerol concentration

Acetic acid, ethanol and glycerol concentrations in the culture media were assayed by HPLC, using a refractive index detector and a Polyspher OA KC (Merck) column. Arabinose was used as the internal standard.

Results and Discussion

Cells of *D. anomala* IGC 5153 were grown in batch cultures with glucose concentrations from 0.1 to 20 % (w/v). Fig. 1 shows representative results of these growth experiments and the values for the concentration of acetic acid, ethanol and glycerol measured at the end of the exponential growth phase in the culture medium are in Table 1. Cells grown in concentrations of glucose higher than 2 % were able to produce high amounts of acetic acid, while in low sugar concentrations the presence of the acid was not detected. Ethanol was detected when the glucose concentration in the culture medium was above 2 %. Additionally, in all growth conditions production of glycerol was not found.

To contribute for the elucidation of the mechanisms underlying the production of acetic acid, we have measured the activity of key enzymes involved in glucose catabolism in extracts of cells grown under different conditions (Fig. 2). In 2 % glucose-grown cells, the activities of the enzymes malate dehydrogenase, NAD^+-acetaldehyde dehydrogenase and acetyl-CoA synthetase

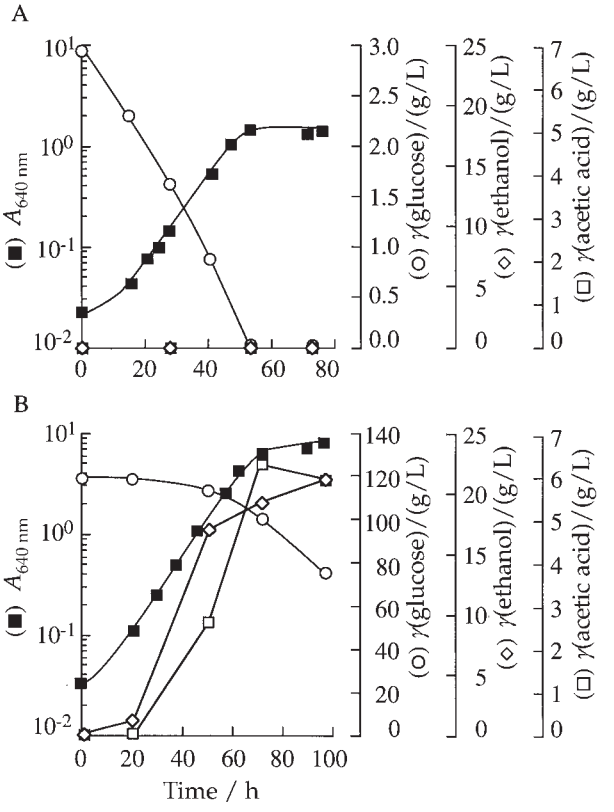

Fig. 1. Growth, glucose consumption and metabolite production by cells of *D. anomala* IGC 5153 grown in mineral medium with glucose at the following concentrations: 0.3 % (A) and 12 % (B); ■, growth measured by absorbance at 640 nm; ❍, glucose concentration; ✧, ethanol concentration; □, acetic acid concentration

Table 1. Specific growth rate (μ_{max}) and production of acetic acid, ethanol and glycerol by cells of *D. anomala* IGC 5153 grown in batch cultures in mineral medium with different initial glucose concentrations; values are mean ±S.D. (*N*=3)

Glucose ratio (w/v) / %	μ_{max} / 1/h	Produced substance**: mmol/g		
		Acetic acid	Ethanol	Glycerol
0.1	0.12 ± 0.01	n.d.	–	–
0.3	0.10 ± 0.02	–	–	–
2.0	0.18 ± 0.01	16 ± 4	26 ± 3	–
12.0	0.09 ± 0.02	18 ± 5	65 ± 6	–
20.0	0.08 ± 0.01	10 ± 3	43 ± 4	–

* not detected

** amount of produced substance at the end of the exponential phase

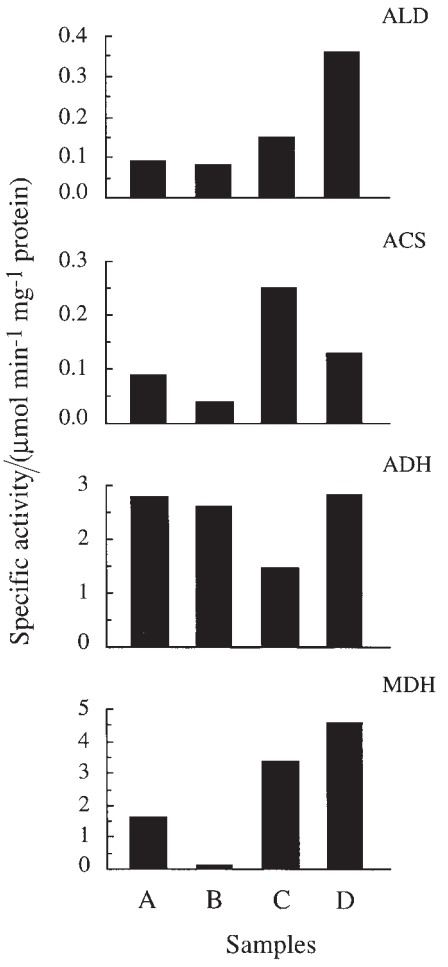

Fig. 2. Specific activities of malate dehydrogenase (MDH), NAD^+-acetaldehyde dehydrogenase (ALD), acetyl-CoA synthetase (ACS) and alcohol dehydrogenase (ADH) in *D. anomala* IGC 5153 grown in mineral medium with different carbon sources; letters on the X-axis indicate the carbon source as follows: A, 0.1 % glucose; B, 2 % glucose; C, 0.5 % ethanol and D, 0.5 % acetic acid. Values are mean of two independent assays

were lower than those measured in 0.1 % glucose-, ethanol- or acetic acid-grown cells. The repression coefficients for the enzymes evaluated, as the ratio of the enzyme activity in ethanol-grown cells (derepression condition) *versus* 2 % glucose-grown cells (repression condition), were the following: for malate dehydrogenase, 18; for NAD^+-acetaldehyde dehydrogenase, 2; for acetyl-CoA synthetase, 6; and for alcohol dehydrogenase, 1. The values for the repression coefficient for malate dehydrogenase, NAD^+-acetaldehyde dehydrogenase and acetyl-CoA synthetase suggested that the respiratory metabolism was not the main pathway involved in glucose catabolism. Indeed, in 2 % glucose-grown cells, a biomass yield of about 0.28 g g^{-1} and a specific glucose transfer rate of 3.53 mmol h^{-1} g^{-1} have been published (*11*) which are consistent with the presence of a fermentative metabolism of the sugar (*7*). On the other hand, 0.1 % glucose-grown cells exhibited a higher activity for the enzymes malate dehydrogenase, NAD^+-acetaldehyde dehydrogenase and acetyl-CoA synthetase comparable to that measured in cells grown on respiratory substrates, namely ethanol and acetic acid. In addition, the absence of ethanol production and the increase of the biomass yield to 0.4 g g^{-1} observed in 0.1 % glucose-grown cells (*11*) pointed to a higher contribution of respiration for the sugar catabolism.

In yeasts, the production of acetic acid has been associated with the oxidation of acetaldehyde. As referred above, the enzyme acetyl-CoA synthetase in *D. anomala* was repressed by glucose and in 2 % glucose-grown cells its specific activity was lower than that measured for NAD^+-acetaldehyde dehydrogenase (Fig. 2). These conditions could favor acetic acid production and its excretion into the medium, once the acetate produced by the acetaldehyde oxidation has not been completely converted to acetyl-CoA. In this respect, a similar mechanism for acetate production was described for *S. cerevisiae* (*7*). On the contrary, in the acetogenic yeast *Brettanomyces abstinens*, the activity of acetyl-CoA synthetase does not seem to account for the blockage of the acetaldehyde oxidative pathway since the activity of the enzyme is not repressed by glucose even in conditions where high amounts of acetic acid are produced (*3*). Thus and in summary, our results showed that cells of *D. anomala* IGC 5153 grown in high glucose concentrations exhibited mainly a fermentative metabolism. The high acetic acid concentrations present in the culture media resulted probably from the insufficient activity of the acetyl-CoA synthetase required for the complete conversion of acetate to acetyl-CoA.

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Biokemijsko proučavanje proizvodnje octene kiseline s pomoću kvasca *Dekkera anomala*

Sažetak

Stanice kvasca *Dekkera anomala* IGC 5153, uzgojene u podlozi s više od 2 % glukoze, sposobne su proizvesti veliku količinu octene kiseline, a u podlozi s manjom koncentracijom šećera nije utvrđena njezina prisutnost. Kad je koncentracija glukoze veća od 20 g/L, nađen je etanol, a glicerol nije utvrđen ni pod kojim uvjetima uzgoja. Izgleda da je fermentativni metabolizam glavni put katabolizma glukoze. Glukoza reprimira enzim acetil-CoA sintetazu, a velike koncentracije octene kiseline, nađene u podlozi, vjerojatno su posljedica nedovoljne aktivnosti acetil-CoA sintetaze potrebne za pretvorbu acetata u acetil-CoA.