

Studies on the Wine Spoilage Capacity of *Brettanomyces/Dekkera* spp.

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Abstract: The presence of *Brettanomyces/Dekkera* in wine during barrel aging is often associated with detrimental organoleptic effects. The mechanisms underlying the capacity of these yeast species to grow and survive in wine with high ethanol levels and low sugar supply were investigated. Ethanol was used by *Brettanomyces bruxellensis* ISA 1791 and *Dekkera anomala* IGC 5153 as the sole carbon and energy source, the growth of the yeasts being dependent on the concentration of alcohol in the culture media. When specific growth rates of both yeasts were plotted against ethanol concentration, at high substrate levels there was a decline in the specific growth rate rather than an asymptotic approach to μ_{\max} according to the Monod-based Bungay equation. Moreover, these yeasts exhibited greater capacity to grow at high ethanol concentrations than *Saccharomyces cerevisiae* IGC 4072. When glucose was used as the sole carbon and energy source, the growth of *B. bruxellensis* showed lower sensitivity to ethanol toxic effects than *S. cerevisiae*. In transport assays carried out with *B. bruxellensis* grown with glucose as the carbon source, the yeast expressed activity for two transport systems for the monosaccharide: facilitated diffusion, specific for glucose and fructose, and H⁺-dependent transport, specific for glucose and galactose and subject to glucose repression. The activity of both transport systems was inhibited noncompetitively by ethanol according to exponential inhibition kinetics, but 12% alcohol reduced sugar transport only by 60%. Findings help explain the ability of *Brettanomyces/Dekkera* spp. to grow and survive under the severe environmental conditions of wine.

Key words: *Brettanomyces/Dekkera* spp., yeast, glucose transport, ethanol tolerance

Yeasts of the genus *Dekkera/Brettanomyces* pose a serious threat to premium wine production. Their detrimental effects on the organoleptic qualities of wines include production of obnoxious flavors and aromas and are responsible for economic loss (Heresztyn 1986, Fugelsang et al. 1993). In particular, these species are reportedly involved in the development of a phenolic character in red wines. Ethylphenols, which may range from a few micrograms to several milligrams per liter, affect wine quality, even in quite small quantities, giving it "animal" phenolic aromas and even barnyard and stable smells at higher concentrations (Chatonnet et al. 1995). In addition, species of both *Brettanomyces* and *Dekkera* are strongly acidogenic and can produce large amounts of acetic acid (Fugelsang et al. 1993, Ciani and Ferraro 1997). Formation of acetate apparently results from the blockage of the acetaldehyde oxidative pathway due to insufficient activity of the enzyme acetyl-CoA synthetase required for the conversion of acetate to acetyl-CoA (Gerós et al. 2000a). These yeast species are not among the dominant organisms in grape juice during fermentation, probably due to their low growth rate, which makes them unable to compete with *Saccharomyces cerevisiae* (Froudiere and Larue 1990, Ciani and Ferraro 1997, Gerós et al. 2000b). However, they are often found in wine, a poor environment for most microorganisms due to minute amounts of sugars and high ethanol levels (Froudiere and Larue 1990). In most instances of *Brettanomyces* in winemaking, such as in barrel aging, the level of avail-

able oxygen is positively correlated with higher growth rates and with an increase in acetic acid production during fermentation or from respiration of ethanol (Ciani and Ferraro 1997). In the present work, physiological studies were performed with the yeasts *Brettanomyces bruxellensis* ISA 1791 and *Dekkera anomala* IGC 5153 to further understand their capacity to tolerate the stressful environment of wine. The capacity of the yeasts to use ethanol as the sole carbon and energy source or glucose in the presence of alcohol was investigated.

Materials and Methods

Strains and growth conditions. *Brettanomyces bruxellensis* ISA 1791, *Dekkera anomala* IGC 5153, and *Saccharomyces cerevisiae* IGC 4072 were maintained on a medium containing glucose (2%, w/v), peptone (1%, w/v), yeast extract (0.5%, w/v), and agar (2%, w/v). Cells were cultivated in 500-mL flasks with 200 mL of synthetic mineral medium containing (NH₄)₂SO₄ (0.5%, w/v), KH₂PO₄ (0.5%, w/v), MgSO₄·7H₂O (0.05%, w/v), CaCl₂·2H₂O (0.013%, w/v) (basal medium), vitamins, trace elements, and glucose or ethanol (van Uden 1967). The vitamin solution consisted of biotin (0.001%, w/v), calcium pantothenate (0.08%, w/v), myo-inositol (4%, w/v), niacin (0.16%, w/v), pyridoxin HCl (0.16%, w/v), and thiamine HCl (0.16%, w/v). Trace element solution A consisted of H₃BO₃ (0.1%, w/v), KI (0.02%, w/v), and NaMoO₄·2H₂O (0.04%). Trace element solution B consisted of CuSO₄·5H₂O (0.008%, w/v), FeCl₃·6H₂O (0.04%), MnSO₄·4H₂O (0.08%, w/v), ZnSO₄·7H₂O (0.08%, w/v), and HCl 0.001 N. Vitamin and trace element solutions were filter-sterilized and added in the proportion of 0.5 mL/L of the basal medium containing the carbon source. All cultivations

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Manuscript submitted March 2003; revised June, September 2003

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were carried out at initial pH values of 3.5 or 4.5 and 26°C, with mechanical shaking (150 rpm). Growth was monitored by measuring absorbance at 640 nm (Spectronic 21, Bausch & Lomb, NY) for determination of specific growth rates (μ). Yield coefficient (Y) was based on dry weight determinations and consumption of glucose. Specific transfer rate of glucose (q) was calculated as the ratio μ/Y .

Yeast specific growth rate with ethanol as growth-limiting substrate. Cells were grown as indicated above in mineral medium supplemented with 0.01 to 9% (v/v) ethanol as the only carbon and energy source. To avoid evaporation of ethanol from the culture media, flasks were closed with rubber stoppers and gas exchange occurred by syringe needles. The dependence of yeast specific growth rate on the concentration of ethanol was analyzed by GraphPad Prism, version 3.0 (GraphPad Software, San Diego, CA), using the following growth kinetic models: (1) $\mu = \mu_{\max} S / (K_s + S)$ (Monod 1942), (2) $\mu = \mu_{\max} S / (K_s + S + S^2/K_i)$ (Andrews 1968), and (3) $\mu = \mu_{\max} S / (K_s + S + S^2/K_i) - K_d S$ (Bungay 1998), where S is the growth-limiting substrate concentration, μ the specific growth rate, μ_{\max} the maximum specific growth rate, K_s the saturation constant, K_i the inhibition constant, and K_d the death term. The best fit for growth kinetics was determined and estimates for the parameters μ_{\max} , K_s , K_i , and K_d were obtained.

Evaluation of ethanol-enhanced cell death. The ability to form colonies was used as the criterion to quantify ethanol-enhanced cell death. Cell suspensions (~0.5 million cells) were incubated in 150 mL of mineral medium supplemented with 10% (v/v) ethanol under shaking in a water bath at 26°C for 1 hr. Flasks were closed with rubber stoppers to avoid evaporation of ethanol from the culture medium. Triplicate samples of 100 μ L were taken at suitable time intervals and spread on the surface of plates of YPD agar. Plates were incubated at 32°C and colonies were counted after visible growth had occurred. The specific death rate (μ_d) was calculated by least-square fitting to the linear part of the semilog survival plot, according the following equation: $\ln N_t/N_0 = \mu_d t$, where N_0 and N_t represent the average of colony forming units at time 0 and time t , respectively.

Effect of ethanol on yeast growth on glucose. Experiments were performed with cells previously grown in mineral medium with 2% (w/v) glucose. The yeasts were collected at midexponential phase and transferred to 250-mL Erlenmeyer flasks containing 100 mL of the same medium to which different ethanol concentrations were added. Specific growth rate was calculated for each situation. The growth inhibition constants were calculated according to the following equation: $\ln \mu^x = \ln \mu^0 - k_i (X - X_{\min})$, where μ^0 is the specific growth rate in the absence of alcohol, μ^x the specific growth rate in the presence of alcohol, X the alcohol concentration, X_{\min} the minimum ethanol concentration above which the toxic effect is measurable, and k_i the exponential inhibition constant.

Estimation of glucose, ethanol, and acetic acid concentration. Glucose, ethanol, and acetic acid concentrations in

the cultures were assayed by HPLC after the cells had been separated by centrifugation. The apparatus used was a Gilson chromatograph with a HyperRez XP Carbohydrate H⁺ column (model 69008-307780, ThermoQuest, Cheshire, UK) maintained at 30°C, with 0.005 N of H₂SO₄ as the mobile phase at a flow rate of 0.5 mL/min. Arabinose was used as an internal standard.

Estimation of initial sugar uptake rates. Cells grown with an initial glucose concentration of 2% and 0.1% (w/v) were harvested at the midexponential growth phase and the late exponential growth phase, respectively, centrifuged, washed twice with ice-cold distilled water, and suspended in distilled water at a final concentration of about 40 mg dry wt/mL. To estimate initial uptake rates of D-[¹⁴C]glucose, 10 μ L of cell suspension was mixed with 30 μ L of 0.1 M potassium phosphate buffer at pH 5.0 in 10-mL conical tubes. After 2 min of incubation at 26°C in a water bath, the reaction was started by the addition of 10 μ L of an aqueous solution of the radiolabeled sugar with 3000 dpm/nmol at the desired concentration. Sampling times were 0, 5, and 10 sec, time periods over which the uptake of labeled sugar was linear. The reaction was stopped by dilution with 4 mL of ice-cold water and the mixtures were filtered immediately through GF/C filters (Whatman, Clifton, NJ). The filters were washed with 8 mL of ice-cold water and introduced into vials containing scintillation fluid OptiPhase HiSafe II (Wallac Scintillation Products, Turku, Finland). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments, Rockville, MD). To assess nonspecific ¹⁴C adsorption, labeled sugar was added after cold water.

Determination of substrate specificity. Inhibition of D-[¹⁴C]glucose transport by unlabeled sugars was assayed by adding simultaneously the labeled and unlabeled substrates. The concentration range of D-[¹⁴C]glucose varied from 0.02 to 0.5 (high-affinity transport system) and 0.5 to 4 mM (low-affinity transport system) and the final concentration of cold substrate was at least 10-fold higher than the K_m value estimated for the carrier.

Accumulation studies. Glucose-grown cells (20 μ L, 40 mg dry wt/mL) were added to 60 μ L of 0.1 M potassium phosphate buffer at pH 5.0. After 2 min of incubation at 26°C, the reaction was started by the addition of an aqueous solution of radiolabeled 3-O-methyl-D-glucose (specific activity = 3000 dpm/nmol) at a final concentration of 1.5 mM. At appropriate times, 10 μ L aliquots were taken from the reaction mixture into 4 mL of ice-cold water and filtered immediately through Whatman GF/C membranes. The filters were washed with 8 mL of ice-cold water and the radioactivity was counted as indicated above. Accumulation of 3-O-methyl-D-glucose was estimated as the ratio between the intracellular and the extracellular 3-O-methyl-D-glucose concentration, using the intracellular volume obtained as indicated below.

Estimation of initial rates of proton uptake. To estimate initial rates of proton uptake upon addition of glucose, a

standard pH meter (PHM 82 Radiometer A/S, Copenhagen, Denmark) connected to a recorder was used as described earlier (Gerós et al. 2000b). The pH electrode was immersed in a water-jacketed chamber with magnetic stirring. A total of 5 mL of cell suspension (about 10 mg dry wt/mL) was added to the chamber. The pH was adjusted to 5.0, and a baseline was obtained. The desired amount of glucose was added, and the subsequent alkalization curve was monitored. The slope of the initial part of the pH trace was used to calculate the initial rates of proton uptake. Calibration was performed with HCl.

Intracellular volume. The intracellular water volume of glucose-grown cells was measured as previously described by De la Peña et al. (1981) and Rottenberg (1979). An intracellular water value of 0.7 ± 0.03 $\mu\text{L}/\text{mg}$ dry wt (mean \pm SE, $n = 3$) was obtained.

Calculation of kinetic parameters. The application of a computer-assisted nonlinear regression analysis (GraphPad Prism, version 3.0) to the data of the initial uptake rates of labeled glucose, as well as of the initial uptake rates of proton disappearance in cell suspensions, allowed us to determine the best fitting transport kinetics and the kinetic parameters. Substrate uptake is presented as the mean of two independent experiments.

Chemicals. D-[U- ^{14}C]glucose (305 mCi/mmol), 3-O-methyl-D-[U- ^{14}C]glucose (98 mCi/mmol), and [^3H]H $_2\text{O}$ (5 Ci/mL) were obtained from Amersham Biosciences (Little Chalfont, UK) and [^{14}C]methoxy-inulin (5.2 mCi/g) from New England Nuclear (Boston, MA). All other chemicals were reagent grade and obtained from commercial sources.

Results

Growth in batch cultures on ethanol. The yeasts *Brettanomyces bruxellensis* ISA 1791 and *Dekkera anomala* IGC 5153 were tested for their capacity to grow, at pH 3.5 or 4.5 and 26°C, in synthetic media supplemented with 0.01 to 9% (v/v) ethanol as the only carbon and energy source. Growth experiments with *Saccharomyces cerevisiae* IGC 4072 were also performed, using the same experimental conditions. Preliminary experiments showed that all the yeast species exhibited similar specific growth rates at pH 3.5 and 4.5; therefore, all subsequent growth experiments were done at pH 4.5, which is the pH value of the complete medium. Monod (1942) was the first to establish a relationship between growth-limiting substrate concentration and specific growth rate by recognizing that it is a rectangular hyperbola and closely resembles that between the velocity of an enzyme-catalyzed reaction and substrate concentration. The maximum specific growth rate (μ_{max}) is

approached when the concentration of the limiting nutrient becomes high enough to saturate the system. This implies that the nutrient ceases to be limiting and that the specific growth rate becomes zero order with respect to the nutrient concentration (Slater 1985). Our results showed that the specific growth rate of *B. bruxellensis*, *D. anomala*, and *S. cerevisiae* is dependent on the extracellular ethanol concentration. However, when μ values were plotted against extracellular ethanol concentration, modified Monod kinetics were obtained, because at high substrate levels there is a decline in the specific growth rate rather than an asymptotic approach to μ_{max} . In media with low ethanol concentrations, *S. cerevisiae* exhibited higher specific growth rates than *B. bruxellensis* (Figure 1); however, this yeast species displayed a higher growth capacity in media with high ethanol levels. *Dekkera anomala* exhibited the same basic behavior as *B. bruxellensis* (Table 1). The application of a computer-assisted nonlinear regression analysis to the experimental data of specific growth rate, according to the Monod

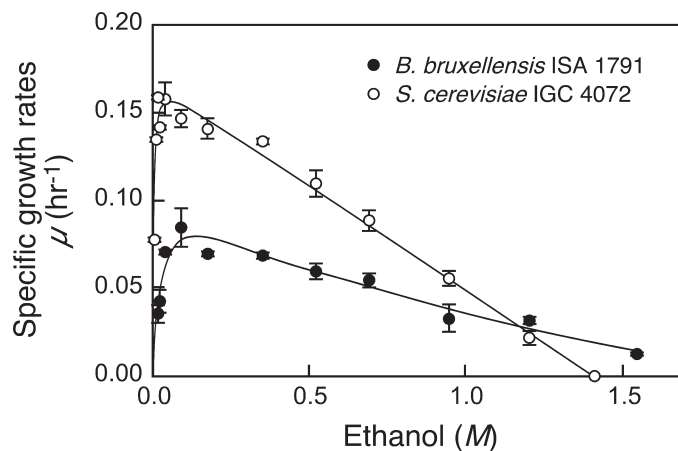


Figure 1 Dependence of the specific growth rates of *B. bruxellensis* ISA 1791 and *S. cerevisiae* IGC 4072 on concentration of ethanol. Cells were grown in defined mineral media at 26°C and pH 4.5, supplemented with ethanol as sole carbon and energy source. Experimental data fitted to the Bungay equation (Bungay 1998) by a computer-assisted nonlinear regression analysis. Vertical bars denote SE, $n = 4$.

Table 1 Values for the Bungay equation parameters (Bungay 1998) estimated by the application of a computer-assisted analysis to the experimental data of specific growth rate of *B. bruxellensis* ISA 1791, *D. anomala* IGC 5153, and *S. cerevisiae* IGC 4072 cultivated in mineral media with different concentrations of ethanol as carbon and energy source.

	Bungay equation values ^{a,b}			
	μ_{max} (hr $^{-1}$)	K_s (M)	K_i (M)	K_d (hr $^{-1}$ M $^{-1}$)
<i>B. bruxellensis</i> ISA 1791	0.101 (0.009)	0.017 (0.004)	1.290 (0.743)	0.020 (0.008)
<i>D. anomala</i> IGC 5153	0.140 (0.008)	0.005 (0.001)	1.564 (0.797)	0.030 (0.010)
<i>S. cerevisiae</i> IGC 4072	0.168 (0.003)	0.002 (0.0003)	>1000	0.118 (0.005)

^a μ_{max} : maximum specific growth rate; K_s : saturation constant; K_i : inhibition constant; K_d : death term.

^bValues in parentheses denote SE, $n = 4$.

(1942), Andrews (1968), and Bungay (1998) equations (see Materials and Methods), allowed us to determine the best fit growth kinetics. For all yeast strains, experimental data displayed a better fit to the Bungay equation, suggesting that the decrease of specific growth rates at higher ethanol concentrations is associated with both growth inhibition and loss of cell viability induced by the alcohol. Values for maximum specific growth rate (μ_{\max}), saturation constant (K_s), inhibition constant (K_i , ethanol concentration necessary to reduce μ to 50% of μ_{\max}), and death term (K_d) estimated by the application of the computer program are shown in Table 1. Taking into account the values obtained for K_d and K_i , the decrease of specific growth rate of *S. cerevisiae* at high ethanol concentrations seems to be primarily due to cell death, and the Bungay equation can be simplified to $\mu = \mu_{\max} S / (K_s + S) - K_d S$. In contrast, high concentrations of the growth substrate primarily inhibit growth in *B. bruxellensis* and *D. anomala*. To demonstrate the involvement of exponential population death in the presence of ethanol, the criterion of ability to form colonies was used. The specific death rate of *S. cerevisiae* (μ_d) was determined at constant temperature (26°C) in cells exposed to 10% (v/v) ethanol, and a value of $\mu_d = 0.15 \text{ hr}^{-1}$ was estimated from the slope of the semilogarithmic survival curve (not shown). Accordingly, from the data of Figure 1 and Table 1, at extracellular ethanol concentration = 1.4 M (8%, v/v) a value of $\mu_d \approx 0.17 \text{ hr}^{-1}$ can be obtained as $\mu_d = \mu_{\max} - \mu$, assuming that the decline of the yeast specific growth rate is exclusively due to cell death.

Growth in batch cultures on glucose. Cells of *B. bruxellensis* were grown in batch cultures with 0.1 to 12% glucose as sole carbon and energy source. Representative results of these experiments, as well as those performed with *S. cerevisiae* IGC 4072, are shown in Figure 2A and Table 2. As expected, *B. bruxellensis* exhibited lower specific growth rates and produced higher amounts of acetic acid and lower amounts of ethanol than *S. cerevisiae*. Similar experiments previously performed with *D. anomala* IGC 5153 (Gerós et al. 2000a) showed that this yeast species exhibits

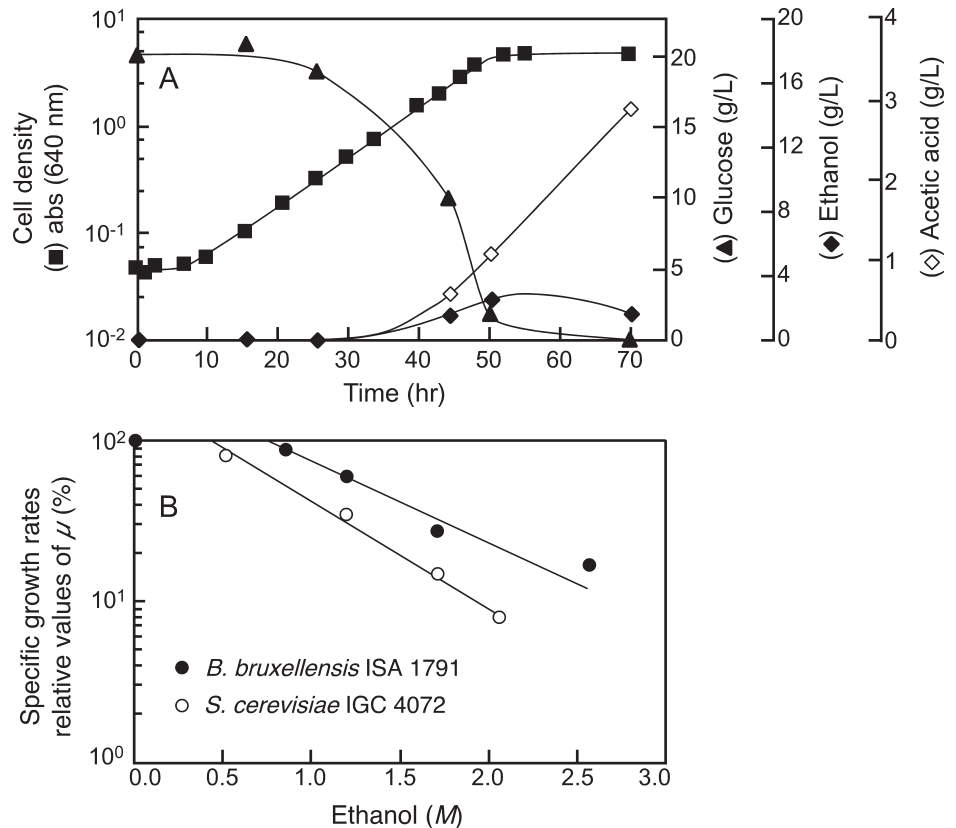


Figure 2 Growth, glucose consumption, and ethanol and acetic acid production by cells of *B. bruxellensis* ISA 1791 cultivated, at 26°C and pH 4.5, in mineral media supplemented with 2.0% glucose as sole carbon source (A). Dependence of the specific growth rates of *B. bruxellensis* ISA 1791 and *S. cerevisiae* IGC 4072 on extracellular ethanol concentration in cells cultivated in mineral media with glucose as indicated in A (B).

Table 2 Growth parameters and metabolite production at the end of exponential phase, in cultures of *B. bruxellensis* ISA 1791 and *S. cerevisiae* IGC 4072 with different concentrations of glucose.

	Growth parameters ^a				Production	
	[Glucose] (% w/v)	μ (hr ⁻¹)	Y (mg dry wt/ mg glucose)	q (mg glucose/h/ mg dry wt)	Ethanol (g/L)	Acetic acid (g/L)
<i>B. bruxellensis</i> ISA 1791	0.1	0.09	0.20	0.44	-	-
	2.0	0.11	0.13	0.82	1.72	2.88
	12.0	0.10	0.20	0.52	1.50	2.45
<i>S. cerevisiae</i> IGC 4072	2.0	0.34	0.16	2.17	2.60	-
	12.0	0.33	0.12	2.80	17.50	0.60

^a μ : specific growth rate; Y: yield coefficient; q: specific glucose transfer rate.

the same basic behavior as *B. bruxellensis* in regard to growth, glucose consumption, and metabolite production. The effect of ethanol on the growth of *B. bruxellensis* on glucose, at pH 4.5 and 26°C, is shown in Figure 2B and compared with the results obtained in growth experiments with *S. cerevisiae*. For both yeast strains, the presence of ethanol in the medium decreased specific growth rate, and the inhibition kinetics obeyed an exponential relationship at ethanol levels above the minimum inhibitory concentration (X_{\min}). The exponential inhibition constant (k_i), the minimum inhibitory concentration, and the concentration necessary to reduce the specific growth rate by 50% (C_{50}) allowed us to calculate ethanol toxicity for both yeast strains (Table 3).

Yeast utilization of glucose requires its transport across the plasma membrane, which, in turn, may constitute an important step for the control of cell growth. In the following approach, glucose transport in *B. bruxellensis* ISA 1791 and its susceptibility to ethanol was examined. The uptake of 0.01 to 10 mM D- 14 C]glucose at pH 5.0, measured in cells grown in a medium with 2% glucose harvested when glucose had fallen to around 1%, obeyed a Michaelis-Menten kinetics (Figure 3A). The application of a computer-assisted simulation to the data was consistent with the presence of a carrier-mediated transport system with the following kinetic parameters: $K_m = 2.32$ mM and $V_{\max} = 1.42$ nmol glucose/sec/mg dry wt. Capacity to transport D-glucose was also ob-

served in cells cultivated with 0.1% sugar collected at the end of the exponential phase when $[\text{glucose}]_{\text{medium}} \ll 0.01\%$; however, the Eadie-Hofstee plot of initial D- 14 C]glucose uptake rates was biphasic (Figure 3B). Furthermore, the computer-assisted nonlinear regression analysis of the data agreed with the presence of two saturable systems for monosaccharide transport. Estimates of the kinetic parameters were $K_m = 0.03$ mM and $V_{\max} = 0.32$ nmol glucose/sec/mg dry wt for the high-affinity component, and $K_m = 3.99$ mM and $V_{\max} = 0.47$ nmol glucose/sec/mg dry wt for the low-affinity component.

Table 3 Effect of ethanol on the growth of *B. bruxellensis* ISA 1791 and *S. cerevisiae* IGC 4072 in the presence of glucose.

Cells were cultivated in mineral medium supplemented with 2% glucose as sole carbon and energy source and transferred to the same medium in the presence of different ethanol concentrations.

	Growth parameters ^a		
	X_{\min} (M)	C_{50} (M)	k_i (M ⁻¹)
<i>B. bruxellensis</i> ISA 1791	0.64	1.34	0.99
<i>S. cerevisiae</i> IGC 4072	0.48	0.92	1.54

^a X_{\min} : minimum inhibitory concentration; C_{50} : concentration necessary to reduce the specific growth rate by 50%; k_i : exponential inhibition constant.

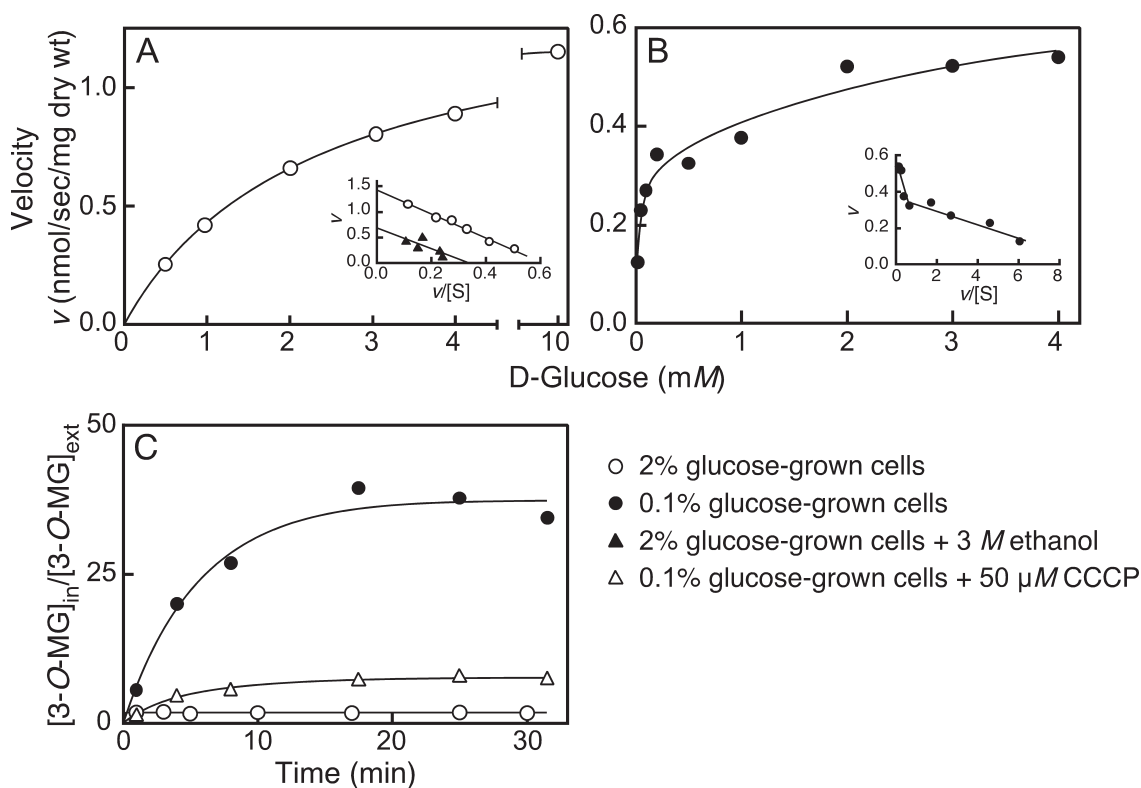


Figure 3 Glucose transport by cells of *B. bruxellensis* ISA 1791 cultivated at 26°C and pH 4.5 in mineral medium with glucose as sole carbon and energy source. Initial uptake rates of D- 14 C]glucose, at pH 5.0, by cells collected at midexponential phase ($[\text{glucose}]_{\text{medium}} \approx 1.0\%$) (A) and at the end of exponential phase ($[\text{glucose}]_{\text{medium}} \ll 0.01\%$) (B) from cultures with initial glucose concentrations of 2.0% and 0.1%, respectively. Inserts: Eadie-Hofstee plots of the initial uptake rates of D- 14 C]glucose in the absence of inhibitors and in the presence of 3 M ethanol. Accumulation of 3-O-methyl-D- 14 C]glucose (3-O-MG), at pH 5.0, by cells cultivated as indicated above (C). Initial extracellular concentration of 3-O-MG 1.5 mM. CCCP was added 10 min before addition of 3-O-MG.

To determine the substrate specificity of the glucose transport systems, inhibition of D-[¹⁴C]glucose uptake by unlabeled sugars was assayed by simultaneously adding the labeled and unlabeled substrate. Results showed that the low-affinity transport system operating in 2% glucose-grown cells was competitively inhibited by D(-)fructose, 2-deoxy-D-glucose, and 3-O-methyl-D-glucose, indicating that they share a common carrier; L-glucose, D(+)galactose, D(+)xylose, D(-)arabinose, mannitol, and the disaccharides sucrose and lactose had no significant effect on the transport of glucose, and thus are apparently not recognized by the glucose transporter. The low-affinity transport system operating in 0.1% glucose-grown cells exhibited the same specificity pattern of the carrier present in 2% glucose-grown cells. Inhibition uptake studies in the higher affinity range indicated that D(+)galactose and 3-O-methyl-D-glucose use the high-affinity transport system; the remaining sugars had no significant effect on the transport of glucose.

The energetics of the monosaccharide transport systems of *B. bruxellensis* ISA 1791 was also studied. The first evidence for the involvement of an H⁺-dependent carrier in glucose transport by the high-affinity transport system was the presence of proton movements associated with glucose uptake by 0.1% glucose-grown cells. Initial velocities of proton disappearance upon addition of 0.02 to 2 mM glucose to weakly buffered cell suspensions, at pH 5.0, are shown in Figure 4A. Proton uptake followed Michaelis-Menten kinetics, and initial velocities were similar to those of D-[¹⁴C]glucose uptake by the high-affinity transport system, suggesting the presence of a monosaccharide-proton symporter with a stoichiometry of 1:1. Conversely, the addition of glucose to cell suspensions of 2% glucose-grown cells was not associated with proton influx (Figure 4B), suggesting that proton-motive force does not constitute the driving force for glucose uptake by the low-affinity transport system. The involvement of a facilitated diffusion was then postulated.

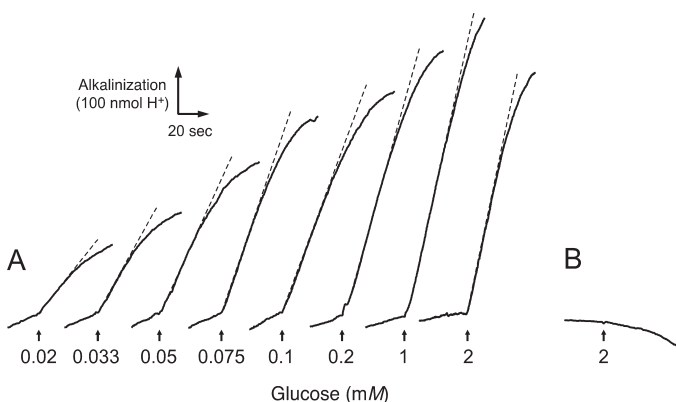


Figure 4 Proton movements, at pH 5.0, associated with the addition of glucose to cell suspensions of *B. bruxellensis* ISA 1791 cultivated at 26°C and pH 4.5 in mineral medium with 0.1% (A) and 2% glucose (B) as indicated in Figure 3.

The transport of the analog 3-O-methyl-D-[¹⁴C]glucose along time, at pH 5.0, in 0.1 and 2% glucose-grown cells of *B. bruxellensis* is shown in Figure 3C. Cells expressing activity only for the low-affinity transport system were not able to accumulate the sugar above levels of diffusional equilibrium. In contrast, 3-O-methyl-D-[¹⁴C]glucose was accumulated to levels of about 30-fold in cells displaying activity for the high-affinity transport system, and the addition of 50 μM of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibited 3-O-methyl-D-glucose accumulation by 80%, corroborating the involvement of an H⁺-dependent monosaccharide transport system.

To ascertain whether glucose transport constitutes a rate-limiting step of *B. bruxellensis* growth on glucose, the growth parameters of the yeast were compared with the maximal capacity of sugar uptake. It was found that the values for the specific glucose transfer rate (*q*) (Table 2) were similar to those of the corresponding V_{\max} of glucose transport, as follows: $q = 1.27$ nmol/sec/mg dry wt and $V_{\max} = 1.42$ nmol/sec/mg dry wt, for 2% glucose-grown cells, and $q = 0.68$ mg glucose/hr/mg dry wt and $V_{\max} = 0.47$ nmol/sec/mg dry wt, for 0.1% glucose-grown cells, suggesting that sugar uptake is an important metabolic step for the control of cell growth.

After characterization of glucose uptake in *B. bruxellensis*, we investigated whether the cells were able to use the sugar in the environmental conditions of wine, which has a high amount of ethanol. The alcohol noncompetitively inhibited D-[¹⁴C]glucose uptake by both low-affinity transport system (Figure 3A) and high-affinity transport system (not shown) and the inhibition kinetics of V_{\max} obeyed exponential relationships at ethanol levels above the minimum inhibitory concentration (not shown). The values for the exponential inhibition constants (k_i) and for the minimum inhibitory concentrations (X_{\min}) were 0.9 M⁻¹ and 1.0 M for the low-affinity glucose transport system and 0.9 M⁻¹ and 0.5 M for the high-affinity glucose transport system, respectively.

Discussion

The presence of minute amounts of unfermented sugar has been found to predispose wine to the growth of *Dekkera/Brettanomyces* species (Chatonnet et al. 1995, Fugelsang et al. 1993). Here, we discuss the capacity of these yeasts to develop in wine with glucose, testing the ability of *Brettanomyces bruxellensis* ISA 1791 to grow in mineral medium supplemented with 0.1 to 12% glucose as the sole carbon and energy source. However, other substrates present in wine can support growth of these yeast species and are presumably associated with their spoilage capacity when no sugar is available. Gerós et al. (2000b) reported that *Dekkera anomala* IGC 5153 is able to grow with 0.1 to 3% (v/v) acetic acid as sole carbon and energy source, over a pH range of 3.5 to 5.5, and the involvement of a monocarboxylate-proton symport on the uptake of the substrate was postulated. Additionally, according to

Fugelsang et al. (1993), ethanol is assimilated aerobically and may represent the sole carbon source for *Dekkera/Brettanomyces*. However, little information is available about the capacity of these yeast species to grow using ethanol when it is present at medium to high levels in wines. The present work showed that *B. bruxellensis* ISA 1791 and *D. anomala* IGC 5153 are able to grow, although slowly, using ethanol as the sole carbon and energy source in synthetic media with high ethanol content, up to 9% (v/v), and that they have low susceptibility to death activation by the alcohol. The decline of specific growth rates of *S. cerevisiae* at high ethanol concentrations appears to be mainly associated with the loss of cell viability, thus, exponential death concurs with exponential growth, validating the Bungay growth kinetics model.

As observed by Chatonnet et al. (1995), the growth of *Brettanomyces/Dekkera* yeasts by fermentation of about 275 mg/L of sugars such as glucose, fructose, and galactose, much less than the average residual sugar level of new red wine at the end of the usual fermentation process, is sufficient to produce undesirable sensory effects. Additionally, the presence of oxygen stimulates the growth and fermentative metabolism of these yeast species (Custers effect), contrary to what is observed in *S. cerevisiae* (Wijsman et al. 1984, Ciani and Ferraro 1997). Our results show that when *B. bruxellensis* ISA 1791 was grown aerobically in mineral medium with glucose as sole carbon and energy source, increasing the sugar concentration in the culture media from 0.1 to 12% did not promote an increase of the specific growth rates. This suggests, according to the Monod growth kinetics model, that the maximum value for the specific growth rate (μ_{\max}) had been achieved. Since low values for biomass yield (Y) were obtained in all growth conditions studied, fermentation was primarily responsible for this catabolism. Regarding the growth studies of *B. bruxellensis* and *S. cerevisiae* in mineral media with glucose in the presence of ethanol, comparison of the values obtained for the inhibition parameters k_i , X_{\min} , and C_{50} suggested that *B. bruxellensis* exhibited lower sensitivity than *S. cerevisiae* to the toxic effects of alcohol. In the case of *B. bruxellensis*, μ decreased about 75% by 12% ethanol, suggesting that the yeast is still able to grow in wines using glucose as a substrate.

Data from transport assays carried out with *B. bruxellensis* cultivated with glucose as the carbon source showed that the yeast has two transport systems for the monosaccharide which could be distinguished by their kinetics, energetics, and specificity: (1) a monosaccharide:H⁺ symporter expressed only when [glucose]_{medium} << 0.01%, with an apparent stoichiometry of 1 sugar:1 proton and (2) a facilitated diffusion transport system operating in cells grown with either high or low glucose levels. The yeast *D. anomala* also appears to adapt its glucose transport systems to the sugar concentration in the culture, a carrier with high affinity being operational when it is grown with a low sugar supply (Gerós et al. 1999). These results seem to be ecologically relevant as the presence of such high-affinity

transport systems would allow *Brettanomyces/Dekkera* species to use minute amounts of unfermented sugars in wine, which could account for their spoilage capacity.

A number of alcohol-sensitive rate processes that underlie the alcohol sensitivity of growth, fermentation, and viability in *S. cerevisiae* and other yeasts have been identified. It has been shown that ethanol inhibits processes of mediated transport and stimulates transport of compounds that cross the membrane by simple diffusion. By increasing transmembrane proton influx, ethanol may induce intracellular acidification (Leão and van Uden 1985). In this work, the effect of ethanol on glucose transport by *B. bruxellensis* seemed to account for the observed inhibition of yeast growth in media with glucose since glucose transport behaved as the growth rate limiting step and the value for the exponential inhibition constant of glucose transport by ethanol was similar to that estimated for the exponential inhibition constant of growth. Even so, since 12% ethanol inhibited glucose transport by the high-affinity transport system only by 60%, the yeast seems to be able to grow in wines using the residual amounts of glucose.

Conclusion

Many studies have been carried out showing a remarkable resistance of *S. cerevisiae* to ethanol. However, *Brettanomyces/Dekkera* species seem better adapted than *S. cerevisiae* to survive in wine, an unpropitious environment for most microorganisms with minute amounts of sugars and high ethanol levels. In the absence of glucose, *B. bruxellensis* and *D. anomala* exhibited a higher growth capacity at high ethanol concentrations, and appeared to have lower susceptibility to death activation by ethanol. The growth of *B. bruxellensis* on glucose showed lower sensitivity to the toxic effects of ethanol. These yeast species are able to express high-affinity monosaccharide transport systems in media with low sugar supplies.

To advance knowledge about the wine spoilage capacity of *Brettanomyces/Dekkera* yeasts, growth experiments were conducted in defined mineral media enabling the precise control of extracellular concentrations of ethanol and glucose. However, since these conditions are somewhat removed from those in a wine ecosystem, complementary studies on the properties of these yeasts in various wines with different ethanol and sugar content would be of great practical value.

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