Journal of Industrial Microbiology & Biotechnology (1997) 18, 379–383

Derepressed utilization of L-malic acid and succinic acid by mutants of *Pachysolen tannophilus*

CJ Harrod[1,2], SB Rodriguez[3,4] and RJ Thornton[1,4]

[1]*Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand;* [3]*Biotechnology Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand*

Utilization of the tricarboxylic acid (TCA) cycle intermediates, L-malic acid and succinic acid, by the yeast *Pachysolen tannophilus* is repressed in the presence of glucose. Strains of *P. tannophilus* containing mutations in two hexokinases and a glucokinase were characterized for growth on glucose plus L-malic acid or succinic acid. Increased specific utilization rates of malic acid and succinic acid in the presence of glucose were observed in mutants containing a lesion in hexokinase A, an enzyme associated with catabolite repression. Such derepressed mutants may have application in winemaking in which utilization of a major grape acid, L-malic acid, is often desirable for acidity reduction.

Keywords: yeast; organic acids; wine

Introduction

The major acids present in grapes and wine are malic acid and tartaric acid. In cool climates, grapes may contain high levels of these acids, which may adversely affect the quality of wine. Traditionally, the growth of lactic acid bacteria which utilize malic acid, the so-called malo-lactic bacteria, has been encouraged in high acid grape juice [8]. Recently, interest in malic acid-utilizing yeasts has increased. *Saccharomyces cerevisiae*, the yeast conducting the alcoholic fermentation in grape juice is a K– yeast, ie it cannot use TCA cycle intermediates as sole carbon and energy source [1]. *S. cerevisiae* strains utilize little of the malic acid present during winemaking [13]. An active malic acid utilizing K– wine yeast, *Schizosaccharomyces pombe*, often generates off-flavors during its growth in wine [16]. A mutant of *Schizosaccharomyces malidevorans* has been isolated which rapidly utilizes malic acid without appreciable sugar utilization but which requires both malic acid and glucose for growth [14]. Microvinification and winery trials of this yeast, along with an *S. cerevisiae* strain to conduct the alcoholic fermentation, have resulted in organoleptically acceptable wines of reduced acidity [17].

Attention is now focusing on the utilization and transport of L-malic acid by K+ yeasts, yeasts which can utilize one or more TCA cycle intermediates as sole carbon and energy source. Glucose and fructose, the major grape sugars, repress the utilization of L-malic acid in the K+ yeasts studied. Specific activities of yeast enzymes can be repressed from four-fold (eg isocitrate dehydrogenase) to 750-fold (eg invertase) in the presence of glucose [6]. Utilization of L-malic acid by the K+ yeasts, *Pachysolen tannophilus* and *Pichia stipitis*, is reduced 55% and 90%, respectively, in the presence of 10% glucose [15]. Corte-Real *et al* [5] reported the isolation of mutants of the K+ yeast *Hansenula anomala* which utilize malic acid rapidly and completely in the presence of 30% glucose. In three K+ yeasts, *H. anomala* [4], *Candida sphaerica* [3] and *Kluyveromyces lactis* [21], dicarboxylic acids, including L-malic acid and succinic acid, are transported by catabolite-repressed carriers. In mutants of *H. anomala* derepressed for malic acid utilization, dicarboxylic acid transport is active in the presence of glucose [5].

Phosphorylating enzymes, kinases, catalyze the first step in the metabolism of glucose and other hexoses. *In Saccharomyces cerevisiae*, two of these hexose kinases, hexokinase PII coded by the *HXK*2 gene and hexokinase PI coded by the *HXK*1 gene, appear to play key roles in glucose repression [10,11]. A third kinase, glucokinase, is specific for glucose and does not appear to be involved in carbon catabolite repression. There is one hexose kinase in the yeast *Schwanniomyces occidentalis*, and it appears to mediate catabolite repression [12]. Like *S. cerevisiae, P. tannophilus* has two hexokinases and a glucokinase [19]. One of these enzymes, hexokinase A, is associated with catabolite repression [18,19]. In this paper, we report on derepressed utilization of L-malic acid and succinic acid by hexokinase mutants of *P. tannophilus*.

Materials and methods

Yeast strains

Pachysolen tannophilus NRRL Y-2460, a wild-type strain, was obtained from A James, NRC, Ottawa, Canada. *P. tannophilus* mutants P510-5A (*glu*1), P509-3C (*hxk*2), P509-1B (*hxk*2 *glu*1), D/X A (*hxk*1 *hxk*2 *glu*1) were derived from *P. tannophilus* NRRL Y-2460 by selection for resistance to 2-deoxyglucose [19].

Correspondence: RJ Thornton, E&J Gallo Winery, PO Box 1130, Modesto, CA 95353, USA
[2]Present address: Thermophile Research Unit, University of Waikato, Private Bag 3105, Hamilton, New Zealand
[4]Present address: E&J Gallo Winery, PO Box 1130, Modesto, CA 95353, USA
Received 4 October 1996; accepted 13 March 1997

Media

Agar indicator media, MMG and MM liquid media were prepared as previously described [14], except that indicator media contained 8% (w/v) rather than 10% glucose. The indicator medium contained 8% glucose, 0.67% (w/v) Difco Yeast Nitrogen Base without amino acids, 0.0022% (w/v) bromocresol green, 2% agar, and one of the following organic acids (1%, w/v): L-malic acid, citric acid, succinic acid or fumaric acid. MMG contained 0.67% Difco Yeast Nitrogen Base without amino acids, 10% D-glucose and 0.3% L-malic acid. MM contained 0.67% Difco Yeast Nitrogen Base without amino acids and 0.3% L-malic acid. In MSG, 0.3% succinic acid was substituted for L-malic acid. The final pH of all media was 3.8. The minimal agar medium contained 0.67% Difco Yeast Nitrogen Base without amino acids, 2% L-malic acid, citric acid, succinic acid or fumaric acid, and 2% agar. The pH of the media was adjusted to 2.5, 3.0, 3.8, 4.0, 5.0 or 6.5 with HCl or KOH prior to addition of agar. For media with pH values of 3.8 and below, agar suspensions were autoclaved separately to prevent hydrolysis.

Culture conditions

Inocula were grown in MMG for 48 h at 30°C. Flasks (1 L) containing 400 ml MMG were inoculated at a concentration of 4.5×10^{-4} mg dry cell mass ml^{-1}. Flasks with fermentation traps were incubated at 25°C with gentle shaking (100 rpm) to keep cells suspended. Samples (10 ml) were removed for A_{600} and pH measurements and, after filtration, were frozen for HPLC analysis. A standard curve of dry cell mass $\times$ A_{600} was generated to obtain cell mass conversion. All fermentations were done in duplicate.

Analyses

Glucose, L-malic acid, succinic acid and fumaric acid concentrations were determined by HPLC. A Brownlee (Norwalk, CT, USA) PPH-224 cation exchange column (Polypore H) and a Brownlee OSS-MP reverse phase column (Spheri 5) were used in series. The mobile phase was 0.0065 M H_2SO_4 at a flow rate of 0.3 ml min^{-1}. The column temperature was 30°C. Refractive index detection was used for glucose analysis and UV (210 nm) detection was used for organic acid analysis.

Specific utilization rates

Specific rates of malic acid, succinic acid and glucose utilization (mmol g^{-1} h^{-1}) were obtained as follows: the amount of substrate utilized between two sampling times (Figure 1a–e) was determined and divided by the average cell mass during that interval. These numbers were plotted against time and the resultant lines were smoothed by eye. The values on the new curve are the specific utilization rates found in Tables 1 and 2.

Results and discussion

Growth of *P. tannophilus* NRRL Y-2460 was tested on minimal agar media containing the dicarboxylic acids, L-malic acid, succinic acid, fumaric acid or citric acid (all at 2%, w/v) as sole carbon source at pH 2.5, 3.0, 3.8, 4.0, 5.0 and 6.5. *P. tannophilus* did not grow on citric acid, in agreement with standard taxonomic texts [2,9] but grew well on the 4-carbon acids at pH values up to 3.8. Growth was reduced at pH 4.0 and was not apparent for 3 days at pH 5.0. No growth occurred on any acid at pH 6.5 although growth on glucose was good at this pH. The decrease in growth on the three organic acids at pH 4–5 and the lack of growth at pH 6.5 may be due to the increased concentration of dissociated acid, which does not pass through the cell membrane as readily as the undissociated form. The pk_{a2} values of L-malic acid, succinic acid, and fumaric acid are 5.1, 5.6 and 4.6, respectively.

The final color of the green indicator plate was used for the preliminary characterization of organic acid utilization by the hexokinase mutants. Indicator medium containing glucose alone turned yellow-green, an acidification, upon growth of the wild-type and mutant strains, P510-5A and P509-3C. The hexose-ATP-phosphorylase specific activity of the latter two yeasts was assayed as 79% and 48%, respectively, of the wild-type activity [19]. By contrast, the mutant strains, P509-1B and D/X A, containing 6% and 0.8% of the wild-type hexose-ATP-phosphorylase activity, respectively [19], did not grow on this indicator medium. On plates containing glucose plus malic, fumaric or succinic acid, the mutants fell into two groups, dependent on the level of glucose phosphorylating activity. The color of the indicator medium remained unchanged after growth of the wild-type, and strains P510-5A and P509-3C, indicating a co-utilization of the two carbon sources. Growth of strains P509-1B and D/X A on the indicator medium, however, resulted in a blue color, a net deacidification of the plate. Thus, the color of the medium, its final pH, was an indicator of a strain's sugar utilization relative to its organic acid utilization.

Different patterns of glucose and malic acid utilization were observed in fermentations by the *P. tannophilus* wild-type strain and mutants (Figure 1). *P. tannophilus* NRRL Y-2460 utilized both glucose and malic acid during both exponential growth and stationary phases in MMG (Figure 1a). In 5 days, *P. tannophilus* utilized 51% of the L-malic acid and 87% of the glucose in MMG. It utilized 76% of the L-malic acid in 5 days when grown on malic acid as sole carbon source (Figure 1b). The kinetics of malic acid and glucose utilization by P510-5A were indistinguishable from the wild-type (data not shown). Decreased glucose utilization by strains P509-3C, P509-1B and D/X A was accompanied by an increased rate of malic acid utilization (Figure 1c–e).

When both glucose and malic acid were co-utilized, as by the wild-type (Figure 1a), the pH of the medium remained constant, as results on the indicator plate had predicted. The acidification by glucose utilization was basically neutralized by the alkalinization of organic acid utilization. When glucose was not utilized, as by the triple mutant, D/X A (Figure 1e) or the wild-type strain growing on malic acid alone (Figure 1b), a simple inverse relationship between malic acid concentration and pH was observed. The growth rates of strains P509-1B (Figure 1d) and D/X A (Figure 1e) decreased at 96 h when the pH was approximately 4.7. Glucose utilization in cultures of strain P509-1B was first detected at approximately 114 h (Table 2), and resulted in a decrease in pH of the medium

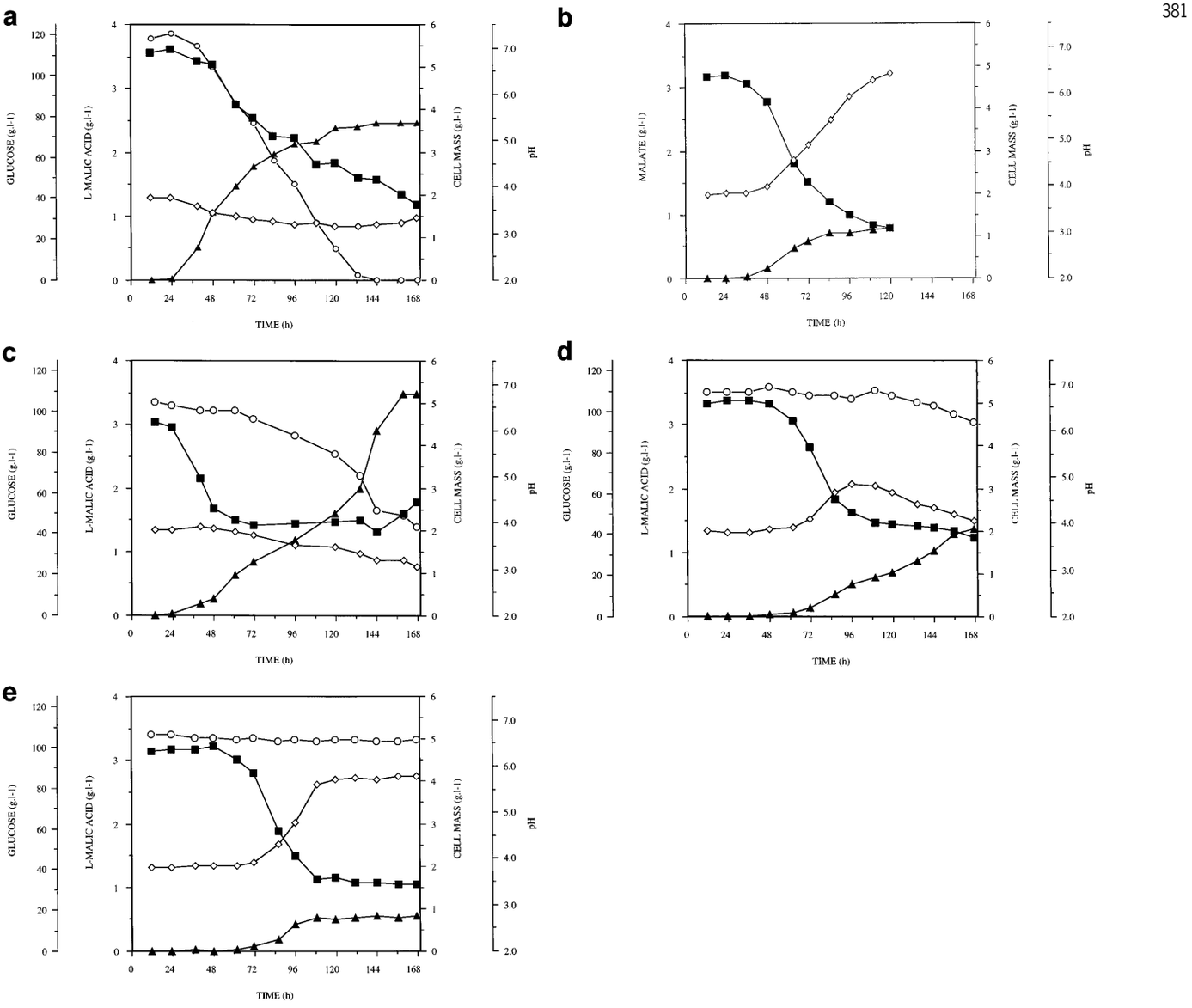

Figure 1 Fermentation of *Pachysolen tannophilus* strains in malic acid-containing medium. Values in graphs are the average of measurements made on duplicate cultures. Glucose, ○; L-malic acid, ■; cell mass, ▲; pH, ◇. (a) Strain NRRL Y-2460 in MMG; (b) strain NRRL Y-2460 in MM; (c) strain P509-3C in MMG; (d) strain P509-1B in MMG; (e) strain D/X A in MMG.

Table 1 Specific rates of L-malic acid and succinic acid utilization (mmol g^{-1} h^{-1})

Time interval (h)	Strain and medium					
	Y-2460 MMG	Y-2460 MM	P509-3C MMG	P509-1B MMG	D/X A MMG	P509-1B MSG
30–32	0.12	3.38	2.98	0	0	0
42–44	0.08	1.27	1.36	0	0	0
54.5–55	0.19	0.99	0.15	2.66	5.32	1.29
66.5–67	0.06	0.36	0.06	2.98	2.30	1.83
78–84	0.04	0.18	0.01	1.14	2.39	0.92
90–91	0.04	0.13	ND	0.24	0.66	0.16
102.5–108	0.04	0.07	0.00	0.10	0.33	0.03

ND, not determined for this interval.

(Figure 1d). This pH decrease correlated with an increased growth rate and the resumption of malic acid utilization. In D/X A cultures, which did not use glucose, the pH increased to 5.7 with consequent cessation of growth and malic acid utilization (Figure 1e). Growth of *P. tannophilus* on malic acid in agar media was dependent on the pH of the medium, with significant inhibition at pH 4 and above. The inhibition of growth observed in malic acid-plus-glucose liquid cultures may be due to the increase in pH.

The results with indicator media suggest that the reduced hexokinase activity may result in derepressed utilization of other dicarboxylic acids. Thus, a malic acid-derepressed mutant and the wild-type *P. tannophilus* were grown on succinic acid and glucose. Utilization of glucose by the wild-type was similar in MMG (Figure 1a) and MSG (Figure 2a); however, there was a net increase in succinic

Table 2 Specific rates of glucose utilization (mmol g^{-1} h^{-1}) in MMG

Time interval (h)	Strain			
	Y-2460	P509-3C	P509-1B	D/X A
30–32	3.94	2.78	0	0
42–44	5.66	2.14	0	0
54.5–55	4.22	1.94	0	0
66.5–67	2.72	0.94	0	0
78–84	2.50	1.23	0	0
90–91	2.22	ND	0	0
102.5–108	2.11	1.01	0	0
114.5–115.25	1.94	ND	1.89	0
126–127.5	1.44	1.52	0.94	0
138–139.5	0.44	1.35	1.33	0
150–152	0	0.87	0.83	0
162–164	0	0.67	0.72	0

ND, not determined for this interval.

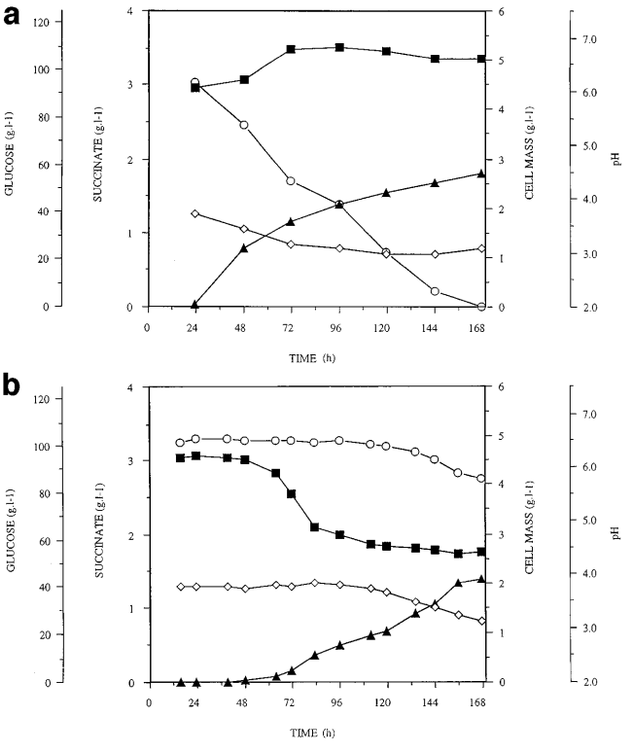

Figure 2 Fermentation of *Pachysolen tannophilus* strains in succinic acid-containing medium. Values in graphs are the average of measurements made on duplicate cultures. Glucose, ○; succinic acid, ■; cell mass, ▲; pH, ◇. (a) Strain NRRL Y-2460 in MSG; (b) strain P509-1B in MSG.

acid. *Saccharomyces cerevisiae* strains produce as much as 2 g L^{-1} succinic acid [20] but only during the exponential phase [7]. Derepressed succinic acid utilization was observed in *P. tannophilus* P509-1B growing in MSG (Figure 2b). The specific rate of succinic acid utilization by strain P509-1B was similar to that of malic acid utilization, indicating a derepressed utilization of both TCA cycle intermediates (Table 2).

Studies of malic acid utilization usually employ high concentrations of glucose, 10–30%, and low concentrations of malic acid, 0.3–0.5% [4,5,15]. In such media, *P. tannophilus* appears to co-utilize malic acid and glucose (Figure 1a); however, when the specific rates of malic acid and glucose utilization (mmol g^{-1} h^{-1}) were calculated, the rate of malic acid utilization was only 4% of the glucose utilization rate at 54–55 h (Tables 1, 2). The specific rates of malic acid and glucose utilization for strain P510-5A, with 79% of the wild-type hexokinase activity, were nearly identical to those of the wild-type (data not shown). Hexokinase activity decreased from 48% in strain P509-3C, to 6% in strain P509-1B, to 0.8% in strain D/X A. Glucose utilization rates for these strains decreased correspondingly (Table 2). The rates of malic acid utilization, however, were similar in strains P509-3C, P509-1B, and D/X A when allowance was made for the differences in lag phase, and were approximately 14 times higher than the wild-type rate (Table 1). Strain P509-3C, containing only the *hxk2* mutation, had similar rates of glucose and malic acid utilization (Tables 1, 2). Thus, it appears that derepression of malic acid and succinic acid utilization is due to altered regulation by the mutated hexokinase A rather than to a change in the glycolytic flux affected by decreased hexose phosphorylating activity. The green color of the indicator plate after growth of strain P509-3C, as opposed to the blue color produced by strains P509-1B and D/X A, is probably due to higher glucose utilization by strain P509-3C which masks the pH increase associated with malic acid utilization.

In *P. tannophilus* P509-1B, which lacks hexokinase A and glucokinase, glucose is utilized by one remaining hexose phosphorylating enzyme, hexokinase B. This enzyme is present at low levels in *P. tannophilus*, and thus the activity of this enzyme is detectable only at high cell densities [19]. In this study, glucose utilization by strain P509-1B (Table 2) was not detected until the cell mass reached approximately 1 g L^{-1}. Malic acid utilization, which was first detected at approximately 48 h, had begun to level off by the time glucose utilization was detected (Figure 1d). Corte-Real *et al* [5] observed that *Hansenula anomala* mutants derepressed for malic acid utilization appeared to metabolize glucose after the malic acid was utilized, the reverse of the wild-type utilization pattern. They termed this phenomenon 'inverse diauxy'. If *H. anomala* has more than one hexose phosphorylating enzyme, as has been found in *S. cerevisiae* and *P. tannophilus*, a 2-deoxyglucose resistant mutant of this yeast may have a remaining hexokinase. This enzyme may be present at a low level as is hexokinase B in *P. tannophilus*. Glucose utilization, especially at the glucose concentration used in the *H. anomala* study, 30%, may not be detected until the culture attains a critical cell mass, and, thus, the phenomenon of inverse diauxy may be questionnable.

Mutants of K+ yeasts such as *P. tannophilus* and *H. anomala* which are derepressed for malic acid utilization have exciting potential for deacidification of musts during winemaking. The indicator plate described in this study has been used to obtain mutants of *P. tannophilus* and another K+ yeast, *Pichia stipitis*, with altered organic acid utilization. A *P. tannophilus* mutant was obtained by spreading UV-mutagenized cells on indicator plates and selecting colonies other than the green wild-type colonies. At a dose yielding 1% survival, 374 white colonies were detected. Fourteen

of these colonies retained the white color when patched onto indicator plates. One of these mutants, when streaked individually on the indicator plate, turned the agar medium blue, indicating derepressed malic acid utilization. This mutant, 335, has an intermediate malate/glucose phenotype: it does not utilize glucose in the presence of malic acid but utilizes less malic acid than strain D/X A. Like strain D/X A, it turns the indicator medium blue. However, unlike strain D/X A and like the wild-type, it turns the citric acid plus glucose plate yellow-green. The potential of using such indicator plates to obtain mutants which perform in various ways to achieve varied winemaking goals is great. The mutants obtained will have to be extensively evaluated through microvinification and small-scale winery trials. Winemaking parameters in addition to sugar/acid utilization, eg the effectiveness of the mutants in the presence of other yeasts, and the possibility of undesirable sensory effects upon the wine, must be considered. A successful conclusion to such trials will put another powerful microbiological tool in the hands of winemakers.

Acknowledgements

CJH acknowledges the award of a Leonard Condell Postgraduate Scholarship which supported this research.

References

1 Barnett JA and HL Kornberg. 1960. The utilization by yeasts of acids of the tricarboxylic acid cycle. J Gen Microbiol 23: 65–82.
2 Barnett JA, RW Payne and D Yarrow. 1990. Yeasts, Characteristics and Identification, p 449, Cambridge University Press, New York.
3 Corte-Real M, C Leao and N van Uden. 1989. Transport of L(–)malic acid and other dicarboxylic acids in the yeast *Candida sphaerica*. Appl Microbiol Biotechnol 31: 551–555.
4 Corte-Real M and C Leao. 1990. Transport of malic acid and other dicarboxylic acids in the yeast *Hansenula anomala*. Appl Environ Microbiol 56: 1109–1113.
5 Corte-Real M, C Leao and N van Uden. 1990. Inverse diauxy in the yeast *Hansenula anomala*: mutants derepressed for malic acid utilization in the presence of glucose. Appl Environ Microbiol 56: 3402–3404.
6 Gancedo JM. 1992. Carbon catabolite repression in yeast. Eur J Biochem 206: 297–313.
7 Heerde E and F Radler. 1978. Metabolism of the anaerobic formation of succinic acid by *Saccharomyces cerevisiae*. Arch Microbiol 117: 269–276.
8 Henick-Kling T. 1993. Malolactic fermentation. In: Wine Microbiology and Biotechnology (Fleet GH, ed), pp 289–326, Harwood Academic, Chur.
9 Kurtzman CP. 1984. Genus 19 *Pachysolen* Boiden et Adzet. In: The Yeasts: A Taxonomic Study (Kreger-van Rij NJW, ed), pp 289–291, Elsevier Science Publishers BV, Amsterdam.
10 Ma H, LM Bloom, Z Zhu, CT Walsh and D Botstein. 1989. Isolation and characterisation of mutations in the *HXK2* gene of *Saccharomyces cerevisiae*. Mol Cell Biol 9: 5630–5642.
11 Ma H, LM Bloom, CT Walsh and D Botstein. 1989. The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. Mol Cell Biol 9: 5643–5649.
12 McCann AK, F Hilberg, P Kenworthy and JA Barnett. 1987. An unusual hexose-ATP-kinase with two catalytic sites and a role in carbon catabolite repression in the yeast *Schwanniomyces occidentalis*. J Gen Microbiol 133: 381–389.
13 Radler F. 1993. Yeasts—metabolism of organic acids. In: Wine Microbiology and Biotechnology (Fleet GH, ed), pp 165–182, Harwood Academic, Chur.
14 Rodriguez SB and RJ Thornton. 1989. A malic acid dependent mutant of *Schizosaccharomyces malidevorans*. Arch Microbiol 152: 564–566.
15 Rodriguez SB and RJ Thornton. 1990. Factors influencing the utilization of L-malate by yeasts. FEMS Microbiol Lett 72: 17–22.
16 Snow PG and JF Gallander. 1979. Deacidification of white table wines through partial fermentation with *Schizosaccharomyces pombe*. Am J Enol Vitic 30: 45–48.
17 Thornton RJ and SB Rodriguez. 1996. Deacidification of red and white wines by a mutant of *Schizosaccharomyces malidevorans* under commercial winemaking conditions. Food Microbiol 13: 475–482.
18 Wedlock DN and RJ Thornton. 1989. A hexokinase associated with catabolite repression in *Pachysolen tannophilus*. J Gen Microbiol 135: 2013–2018.
19 Wedlock DN, AP James and RJ Thornton. 1989. Glucose negative mutants of *Pachysolen tannophilus*. J Gen Microbiol 135: 2019–2026.
20 Whiting GC. 1976. Organic acid metabolism of yeast during fermentation of alcoholic beverages. J Inst Bew 82: 84–92.
21 Zmijewski MJ and AM MacQuillan. 1975. Dual effects of glucose on dicarboxylic acid transport in *Kluyveromyces lactis*. Can J Microbiol 21: 473–480.