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A novel methodology independent of fermentation rate for assessment of the fructophilic character of wine yeast strains

T. Liccioli · P. J. Chambers · V. Jiranek

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Abstract The yeast *Saccharomyces cerevisiae* has a fundamental role in fermenting grape juice to wine. During alcoholic fermentation its catabolic activity converts sugars (which in grape juice are a near equal ratio of glucose and fructose) and other grape compounds into ethanol, carbon dioxide and sensorily important metabolites. However, *S. cerevisiae* typically utilises glucose and fructose with different efficiency: glucose is preferred and is consumed at a higher rate than fructose. This results in an increasing difference between the concentrations of glucose and fructose during fermentation. In this study 20 commercially available strains were investigated to determine their relative abilities to utilise glucose and fructose. Parameters measured included fermentation duration and the kinetics of utilisation of fructose when supplied as sole carbon source or in an equimolar mix with glucose. The data were then analysed using mathematical calculations in an effort to identify fermentation attributes which were indicative of overall fructose utilisation and fermentation performance. Fermentation durations ranged from 74.6 to over 150 h, with clear differences in the degree to which glucose utilisation was preferential. Given this variability we sought to gain a more holistic indication of strain performance that was independent of fermentation rate and therefore utilized the area under the curve (AUC) of fermentation of individual or combined sugars. In this way it was possible to rank the 20 strains for their ability to consume fructose relative to glucose. Moreover, it was shown that fermentations performed in media containing fructose as sole carbon source did not predict the fructophilicity of strains in wine-like conditions (equimolar mixture of glucose and fructose). This work provides important information for programs which seek to generate strains that are faster or more reliable fermenters.

Keywords Glucose · Fructose · Fermentation progress · Strain comparison · Composite trapezoid rule

Abbreviation
AUC Area under the curve

Introduction

When exposed to mixtures of glucose and fructose, as occurs during the fermentation of grape juice into wine, *Saccharomyces cerevisiae* utilises these sugars at different rates [2, 18]. As a result the ratio between the concentration of glucose and fructose changes, so that late in fermentation fructose becomes the predominant sugar. This is reported to be one of the causes of arrested or so-called stuck fermentation [13]. Attempts to restart the fermentation through re-inoculation with fresh but nonetheless glucophilic cultures are challenging [7]. Regardless of the cause of stuck fermentation, excess residual sugar is undesirable, as it puts the palate of the wine out of balance. Furthermore, the fact that fructose predominates compounds the problem, since fructose is sweeter than glucose [20].

T. Liccioli · V. Jiranek (✉)
School of Agriculture, Food and Wine,
The University of Adelaide, PMB 1,
Glen Osmond, SA 5064, Australia
e-mail: vladimir.jiranek@adelaide.edu.au

T. Liccioli · P. J. Chambers · V. Jiranek
Wine Innovation Cluster, Adelaide, SA, Australia

P. J. Chambers
The Australian Wine Research Institute,
PO Box 197, Glen Osmond, SA 5064, Australia

In seeking the precise basis for differences in the rate of glucose versus fructose utilisation, steps in the metabolism of hexoses prior to the formation of fructose 1,6-bisphosphate are implicated. In particular this includes the systems for sensing of extracellular sugars, their transport across the plasma membrane and phosphorylation as part of the first steps of glycolysis. Plasma membrane sensor proteins have been identified which show affinity for glucose, with at least one exhibiting different affinity for glucose and fructose [30, 31]. The presence of sensors specific for fructose has not been reported [2]. Phosphorylation of internalised glucose and fructose occurs via the hexokinases Hxk1 and Hxk2, albeit at different efficiencies, with glucose additionally being acted upon by glucokinase Glk1 [2, 15, 34]. Since the phosphorylation capacity of these enzymes exceeds the amount of sugar transported into the cell, it would appear that this enzymatic process is not the basis for differences in the utilisation of glucose compared with fructose. However, a recent study [3] demonstrated that over-expression of hexokinases could in fact alter the rate of fermentation. On the other hand, the hexose transport system has been clearly shown by many authors to be a critical point in determining fermentation rate.

In *S. cerevisiae*, hexose uptake is largely mediated by facilitated diffusion [5], where 20 genes (*HXT1* to *HXT17*, *GAL2*, *SFN3* and *RGT2*) encode the related proteins. Despite such plurality, only those transporters encoded by *HXT1* to *HXT7* appear to be of importance for glucose and fructose utilisation under fermentation conditions [9, 10, 12, 25, 26, 29]. Some of these latter HXT transporters have relatively low affinity (high K_m) for hexose, whilst others are considered high affinity (low K_m), and all have the ability to transport both glucose and fructose. Importantly, whether they are high- or low-affinity systems, all have a greater affinity for glucose than for fructose. The expression of each HXT gene is regulated by environmental factors, especially the extracellular hexose concentration [25]. The high-affinity transporters are induced when the glucose concentration is low (~1–4 mM or 0.18–0.72 g/l) and are repressed when the concentration of glucose is higher [8]. Conversely, low-affinity carriers are induced by high glucose concentrations (~50–100 mM or 9–18 g/l) if not constitutively expressed. HXT3, a low-affinity transporter, is considered key to determining glucose transport and, therefore, could play a fundamental role in the different rates of glucose and fructose utilisation [15]. Further work is required to define this role.

Beyond affinity differences, other factors such as nitrogen availability and response to ethanol may be of importance. Ethanol is known to have protein denaturing properties and disrupts plasma membrane components [4, 35]. Due to increases in membrane permeability and passive proton flux upon ethanol exposure, damage to intracellular enzymes and structures might also occur. Berthels et al. [2] observed that a high ethanol concentration inhibited sugar utilisation, but to different extents for glucose and fructose. This led them to hypothesise that the glucose utilisation capability was more robust than fructose utilisation. Moreover ethanol is able to shift the tautomeric equilibrium of fructose from the readily transported pyranose form to the furanose form. Thus, unlike glucose, which is entirely in the transportable pyranose form, typically only 70% of fructose takes the pyranose form, and less in the presence of ethanol. Thus, not only is there a discrepancy between glucose and fructose transport, but it also changes during the progress of fermentation [2] and may be part cause and part effect of different ethanol tolerance of various strains.

A further possibility is linked with nitrogen utilisation and availability. Sugar transporters have been shown to be turned over quickly ($t_{1/2} \approx 5$ h) compared with other proteins [33], thereby creating a demand for active protein synthesis and assimilable nitrogen. In every case of nitrogen supplementation to nitrogen-starved cultures, fructose consumption was enhanced to a greater extent than glucose [2]. Different strains have been shown to utilise nitrogen to different extents [17]. Perhaps therefore, as fermentation progresses, strains with higher nitrogen demands experience greater or earlier restrictions on assimilable nitrogen availability and thus their ability to maintain fructose transport, thereby resulting in a higher rate of glucose transport.

Available evidence affirms that hexose utilisation depends on numerous environmental and biological parameters. Thus determining the precise basis for the glucose/fructose discrepancy in order to target efforts to reduce such differences and presumably improve strain performance and wine composition is difficult. In this study 20 commercially available wine strains were chosen for characterisation of their fructose utilisation proprieties. Such data were sought so as to begin to determine the relationship between abilities regarding utilisation of individual sugars and relative fermentative performance. Fermentations were conducted in a chemically defined grape juice medium (CDGJM) [16] with two different sugar compositions. Either an equimolar mix of glucose and fructose or else a medium containing fructose as the only sugar were used. Various parameters related to total fermentation duration and kinetics of fructose and/or glucose utilisation were measured and the resulting values compared though mathematical calculations to identify relationships.

Materials and methods

Strains and maintenance

A total of 20 commercial strains of *S. cerevisiae* were selected as representative of commonly used wine yeast

and, where possible, chosen with consideration of published information about their ability to consume fructose compared with glucose [2, 15]. The strains used were: B, UCD522, CRU-BLANC, PRIMEUR, AWRI 350, AWRI 796, AWRI 1503, ELEGANCE (AB Mauri, Sydney, Australia); EC1118, V1116, D254, W27, BM45, SYRAH, BORDEAUX RED, S6U, UVAFERM 43 (Lallemand, Montreal, Canada); VIN13, NT202 (Anchor Yeast, Cape Town, South Africa), FERMICHAMP (DSM, The Netherlands). Strains were collected aseptically from active dried commercial preparations, re-hydrated in sterile water (20 min) and inoculated into YEPD medium (20 g/l D-glucose, 10 g/l yeast extract, 20 g/l Bacto peptone) in a flask (air:liquid ratio >66%) before overnight incubation at 28°C with shaking at 180 rpm. Cultures were then streaked onto YEPD agar plates and grown overnight at 28°C to check for purity. Multiple representative colonies were inoculated into 25 ml YEPD broth and grown as above. These served as starter cultures for the fermentation experiments detailed below or, with the combination of 1 ml culture with 0.5 ml sterile 80% (v/v) glycerol, enabled long-term storage at −80°C.

Fermentation experiments

Starter cultures were used to generate pre-cultures, which in turn were used to inoculate fermentation experiments as detailed elsewhere [22]. For each of the 20 strains, fermentations were performed in order to define their sugar utilisation and fermentation kinetics. To do this, two different formulations of CDGJM were used. The first was representative of a typical grape juice in that glucose and fructose were supplied in equimolar amounts to a combined total of 230 g/l. For the second condition an equivalent amount of sugar was supplied but as fructose only. In each case 600 mg N/l (as amino acids [16]) was used, and triplicate fermentations were incubated at 28°C with shaking at 160 rpm.

Fermentation progress was estimated from the Brix value of clarified (14,000 rpm, 2 min) culture samples and fermentation completion (<2.5 g/l) determined using Clinitest® tablets (Bayer). Supernatants were stored at −20°C for subsequent determination of residual sugar content by an enzymatic method [6] adapted for 48-well plates.

Analyses

For every strain, the values obtained from the determination of individual sugars in each medium were considered separately. Four datasets with regard to sugar concentration during the progress of fermentation were obtained, i.e. for glucose, fructose and glucose + fructose in the mixed sugar condition as well as fructose from the fructose-only media. These datasets were used to plot the respective sugar utilisation curves for each strain. In this way, 20 charts were obtained, each containing four areas delimited by the fermentation curves for the four sugars or combinations (Fig. 1). The values of these areas were calculated using the composite trapezoid rule (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA, USA), a numerical integration method for approximating an integral or AUC where the function of the curve is unknown. The value of the AUC is returned in units of the *X* axis times the units of the *Y* axis. Statistical analysis of the data [one-way analysis of variance (ANOVA) with Dunnett and Tukey's multiple-comparison post-test analysis] was performed with the same software.

Results

Fermentation duration

The times required for complete utilisation of glucose and/or fructose were defined for 20 commercial strains of yeast and are summarised in Table 1. For most strains, fermentation of each medium was completed (i.e. <2.5 g/l residual sugar) in less than 150 h. The only exception was Maurivin strain B, for which the mixed sugar fermentation required 151 h and the fructose-only condition failed to complete within this preset maximum duration. In every case the utilisation of fructose lagged behind that of glucose. Similarly, fermentation of fructose took longer than the fermentation of an equivalent amount of mixed sugars. Glucose depletion from the mixed sugar condition occurred in between 60 h (UCD 522) and 115 h (B), whereas fructose depletion occurred within 75 h (UCD 522) and 151 h (B). Since fructose depletion always took longer than that of glucose, the time for the former to occur also equated to the total duration of the mixed sugar fermentations. By comparison, where complete utilisation of 230 g/l fructose was seen, a total of between 94 h (UCD 522) and 134 h (BM 45) was required for this eventuality. Thus the complete fermentation of the fructose-only medium took longer than the equivalent mixed sugar fermentation. These observations confirm the apparent glucophilicity of *Saccharomyces* yeast, and suggest that utilisation of fructose is the rate-limiting step and that the time taken for this defines the total duration of fermentation.

The inclusion of a medium containing fructose as the only sugar provided an opportunity to determine whether this condition was predictive of fructose utilisation capabilities in the mixed sugar medium. The duration of fructose-only fermentation was not strongly correlated ($R^2 = 0.524$) with the time required for fructose depletion from the mixed sugar medium. This indicates that the duration of utilisation of fructose as sole carbon source is not necessarily a good predictor of the duration of fructose utilisation in the presence of glucose.

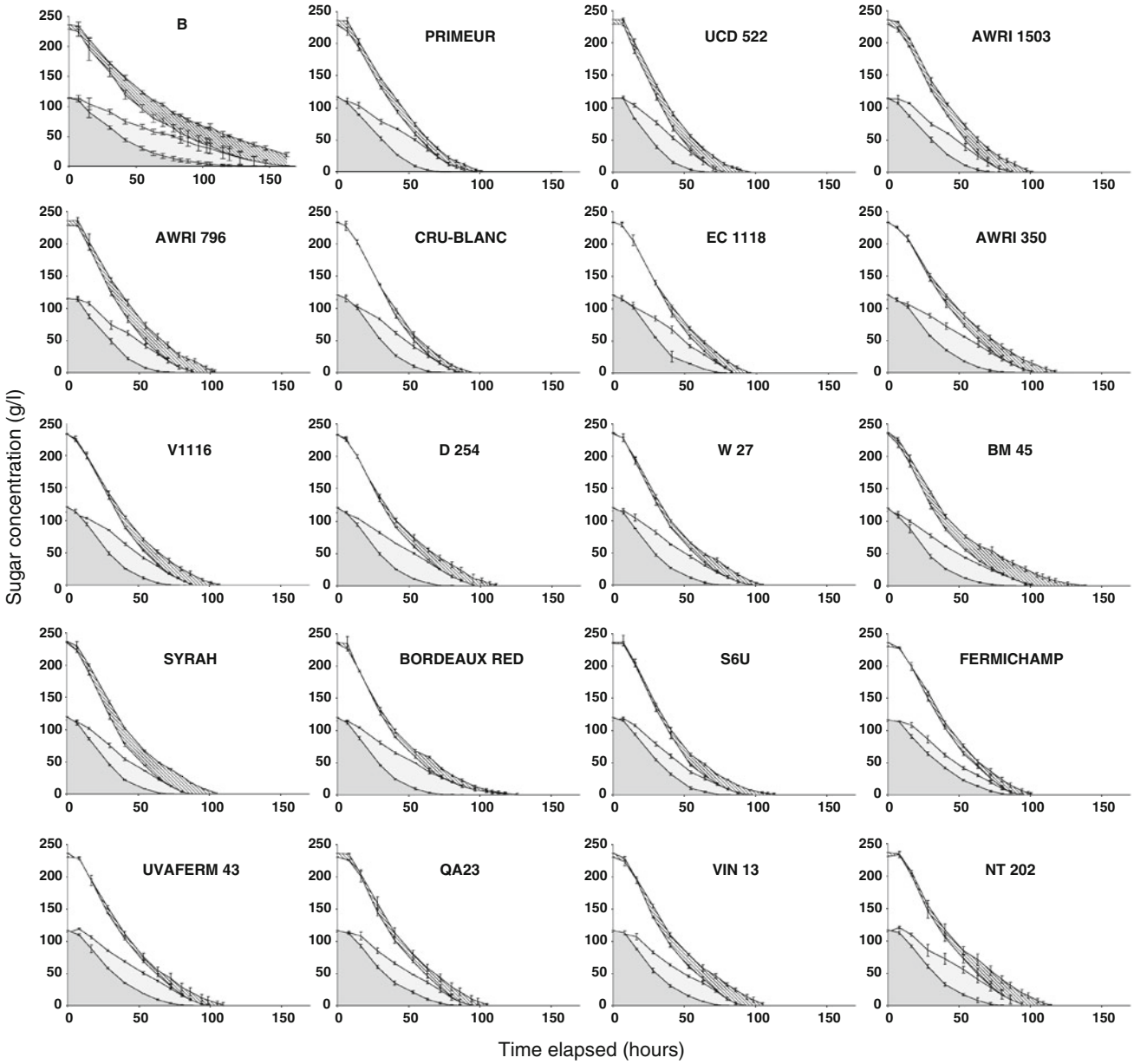

Fig. 1 Composite plots showing sugar utilisation profiles of 20 commercial wine strains in two growth media. Yeast were grown in CDGJM containing either 230 g/l fructose only (*top curve* in each plot) or 115 g/l each of glucose and fructose (1:1; *bottom* three curves in each plot). Curves are derived from the mean of triplicate fermentations with *error bars* indicating standard deviation. Moving from top to bottom, the curves correspond to: (1) fructose fermentation in a fructose-only medium, (2) combined glucose and fructose fermentation from the mixed sugar medium, (3) fructose fermentation from the mixed sugar medium and (4) glucose fermentation from the mixed sugar medium. The hatched area between the *top curve* (fructose fermentation in fructose-only medium) and the *curve below* (combined glucose and fructose fermentation from the mixed sugar medium) highlights the difference in total fermentation profile between the two media. The area corresponding to glucose fermentation from the mixed sugar medium is highlighted in *dark grey*, while the extent to which fructose utilisation from the mixed sugar condition was delayed compared with glucose is highlighted in *light grey*. ± Standard deviation indicated as *bars*

Since fermentation duration alone did not fully describe the performance of individual strains, other features of the pattern of sugar utilisation were sought by plotting the utilisation data (Fig. 1). Several points are evident when the data are presented graphically. Firstly, all fermentations showed some degree of initial lag, followed by an extended period of rapid fermentation, before a progressive slowing toward the complete catabolism of sugars. Thus, these periods would collectively and to different degrees contribute to the overall times required for catabolism of individual sugars. Secondly, differences existed between strains in terms of the extent to which the pattern of utilisation of a given

Table 1 Duration of sugar catabolism and fermentation for 20 commercial yeast strains during growth in media containing glucose and fructose or only fructose to total concentration of 230 g/l

Strain	Duration (h)					
	Glucose (mixed sugars)		Fructose or total sugars (mixed sugars)		Fructose (fructose only)	
	Average	SD	Average	SD	Average	SD
B	115.3	12.5	151.3	20.2	DNC[a]	–
BORDEAUX RED	73.0	0.0	114.5	5.2	120.3	4.9
AWRI 350	77.2	4.9	101.0	0.0	115.3	3.7
BM 45	75.5	4.3	99.2	2.3	134.3	7.2
UVAFERM 43	80.0	0.0	97.0	2.6	106.3	2.3
W 27	73.0	0.0	96.5	0.0	104.5	0.0
D254	71.5	0.0	95.0	0.0	109.5	2.6
PRIMEUR	70.0	0.0	94.3	4.6	101.0	0.0
FERMICHAMP	80.0	0.0	94.0	0.0	99.8	1.1
QA23	80.0	0.0	94.0	0.0	102.8	3.7
VIN 13	72.0	0.0	94.0	0.0	105.0	0.0
NT 202	77.3	4.6	94.0	0.0	112.3	2.9
S6U	73.0	0.0	93.5	5.2	110.0	2.6
SYRAH	64.5	0.0	87.5	0.0	104.5	0.0
V1116	71.5	0.0	86.5	0.0	104.7	3.2
AWRI 796	67.7	4.0	86.0	1.7	102.3	1.1
CRU-BLANC	66.2	4.6	85.2	2.3	95.0	0.0
AWRI 1503	67.7	4.0	84.0	5.2	99.7	2.3
EC1118	71.5	0.0	82.5	0.0	95.0	0.0
UCD 522	60.3	4.6	74.6	4.0	94.3	4.6

Values are the average of triplicate fermentations

[a] DNC, fermentations did not complete

sugar(s) reflected that of another sugar(s). Such differences are highlighted by shading of the area below each curve which distinguishes it from the adjacent curves (Fig. 1). Using this approach and ignoring total fermentation duration, it is evident that strains differ in the extent to which they preferentially utilised glucose compared with fructose in the mixed sugar condition. Consequently, FERMICHAMP has the smallest discrepancy between the two curves (Fig. 1, light gray area), whereas strains such as PRIMEUR, AWRI 796 and BORDEAUX RED appear to have the largest discrepancy. The other key observation is that the difference between the profile for the combined catabolism of glucose and fructose compared with the pattern of fructose utilisation from the single sugar medium also highlights differences between strains (Fig. 1, hatched area). Accordingly, strains such as AWRI 796, SYRAH and BM45 have a large discrepancy, while FERMICHAMP, CRU-BLANC and UVAFERM 43 do not (Fig. 1).

Application of the composite trapezoid rule

Casual observation suggests that there are no examples of particularly anomalous behaviour amongst the strains; that is, there are no instances where a given fermentation duration is in fact not the result of a relatively consistent progression of fermentation, but instead the result of a protracted commencement followed by a very rapid completion. However, the validity of this notion was best assessed through a closer, more quantitative, examination of the data. For this reason much of the subsequent analysis and strain comparison considers the AUC of utilisation of each sugar as determined using the composite trapezoid rule (Table 2).

If, in fact, the rate of fermentation was consistent, then there should be a relationship between the AUC of utilisation for that particular sugar and the time taken for the sugar to be depleted. This was not always the case (note that Maurivin strain B was excluded from this comparison as it was a clear outlier). Thus a comparison of the AUC of glucose (Table 2) was somewhat correlated to the time to glucose depletion ($R^2 = 0.715$). However, the correlation for the analogous values for fructose in the mixed sugar condition yielded an R^2 value of 0.645. These findings are explained by the fact that, in the latter case, there are examples of strains (e.g. BORDEAUX RED and NT202) with similar AUC (i.e. 5,833 and 5,918) but markedly different times for fructose catabolism (i.e. 115 and 94 h). The reverse was also evident, that is, similar durations of fructose utilisation (i.e. 101 and 99 h) but different areas under

Table 2 Area under the glucose and fructose utilisation curves for fermentations by 20 commercial yeast strains of media containing mixed sugars (glucose and fructose) or only fructose to total concentration of 230 g/l

Strains	Area under the fermentation curve (trapezoid rule)									
	Glucose (mixed sugars)		Fructose (mixed sugars)		Glucose area:fructose area (mixed sugars)	Total sugars (mixed sugars)		Fructose (fructose only)		Total sugars area (mixed sugars): fructose area (fructose only)
	Average	SD	Average	SD		Average	SD	Average	SD	
B	4,677	384	8,186	828	0.57	12,864	1,211	DNC[a]	–	–
AWRI 350	3,861	77	6,015	250	0.64	9,876	324	11,123	252	0.89
NT 202	3,743	180	5,918	303	0.63	9,662	481	11,123	572	0.87
UVAFERM 43	3,732	77	5,731	88	0.65	9,463	159	10,201	468	0.93
QA 23	3,844	111	5,548	261	0.69	9,392	371	10,550	565	0.89
FERMICHAMP	4,047	116	5,317	91	0.76	9,364	184	10,108	184	0.93
BORDEAUX RED	3,336	65	5,833	64	0.57	9,169	129	10,026	122	0.91
D 254	3,404	52	5,535	64	0.61	8,939	109	9,937	352	0.90
VIN 13	3,560	77	5,363	68	0.66	8,923	140	10,311	180	0.87
PRIMEUR	3,426	45	5,476	157	0.63	8,902	159	10,082	136	0.88
EC 1118	3,646	73	5,201	216	0.70	8,847	146	9,456	111	0.94
S6U	3,650	16	5,180	123	0.70	8,830	110	9,857	53	0.90
W27	3,400	55	5,403	84	0.63	8,804	139	9,709	105	0.91
BM 45	3,385	113	5,405	76	0.63	8,790	127	11,065	330	0.79
V1116	3,410	95	5,201	46	0.66	8,611	98	9,789	289	0.88
CRU-BLANC	3,516	92	5,052	107	0.70	8,568	178	8,946	119	0.96
AWRI 796	3,297	7	5,135	267	0.64	8,432	272	10,227	495	0.82
AWRI 1503	3,394	8	5,017	252	0.68	8,411	244	9,944	224	0.85
SYRAH	3,216	25	4,832	20	0.67	8,048	25	9,915	27	0.81
UCD 522	3,001	70	4,650	191	0.65	7,651	258	9,067	179	0.84

Values are the average of triplicate fermentations

[a] DNC, fermentations did not complete

the corresponding fructose utilisation curve (6,016 and 5,404) for AWRI 350 and MB45, respectively. These examples highlight the complexity of fermentation phenotypes and the difficulty of comparing strains. When considering the combined catabolism of glucose and fructose from the mixed sugar fermentation, a poor correlation ($R^2 = 0.461$) between the value for the AUC and the total duration of catabolism was again observed, as was the case for the fructose-only condition ($R^2 = 0.482$). Given these obvious complexities, we therefore suggest that, compared with duration alone, the area under the sugar utilisation curve provides a more complete account of sugar utilisation, as it incorporates trends occurring during fermentation.

Utilisation of fructose compared with glucose

Of key interest in this study was the manner in which the kinetics of fructose utilisation influenced the overall fermentation performance of individual strains. For this reason we sought to identify a basis for comparison of strains which was independent of overall fermentation duration. As such we compared each strain in terms of the ratio between the areas under each of the glucose and fructose utilisation curves (Table 2). All strains exhibited a glucose:fructose ratio which was markedly less than 1, clearly demonstrating the ability of these strains to preferentially utilise glucose over fructose. The strains ranged in terms of this ratio, such that BORDEAUX RED exhibited a value of 0.57 compared with 0.76 for FERMICHAMP.

The ratio of AUC of the utilisation of glucose compared with fructose in the mixed sugar condition is proposed to give an indication of the relative glucophilicity (increased fructose or poor glucose consumption) of individual strains. Based on this concept, we averaged the values of the ratio between glucose and fructose areas for the 19 strains (B excluded) and used the resulting average as the reference ratio (0.66). Thus, it was possible to rank all the strains based on their individual glucose:fructose ratio deviation from the mean of all ratios (Fig. 2). Two groups of strains can be identified: the first represents the more fructose-efficient strains (negative values), and the second represents the less fructose-efficient strains (positive values). In this

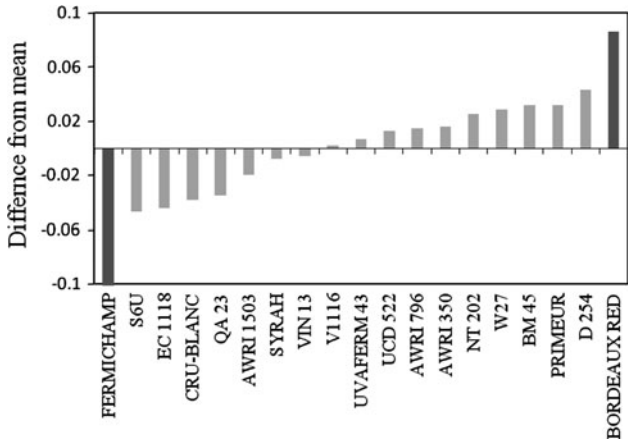

Fig. 2 Differences from the mean (baseline) of ratios between AUC of glucose and fructose in the mixed media conditions (as reported in Table 2). The values were calculated with one-way ANOVA Dunnett's multiple-comparison post-test at 95% confidence interval. FERMICHAMP and BORDEAUX RED (in *dark*) were the only two strains showing significant differences from the average

way FERMICHAMP appears to be either more fructophilic or less efficient at utilising glucose. Additionally, all strains were compared against each other to determine any significant differences (Table 3). Most strikingly, FERMICHAMP and BORDEAUX RED were the two strains with most significant differences from the other strains.

As was shown for the duration of fermentation, further evidence for the poor relationship between performances in one condition (medium) compared with another is provided by comparison of the ratio of the AUC of utilisation of both glucose and fructose compared with fructose alone (Table 2). Such a comparison yielded a correlation of $R^2 = 0.449$. Further consideration of strain performance was therefore limited to a comparison of the relative utilisation of glucose and fructose in the mixed sugar condition.

Discussion

Developing a valid method to assess glucose and fructose utilisation during alcoholic fermentation is not a straightforward matter. Several authors have proposed criteria for this, but what is clear, and supported by our data, is that the method must be independent of the overall rate or duration of the fermentation. Also, to simplify its application, especially for comparison of many strains, the approach must minimise the need for real-time analysis and, given the dynamic nature of fermentation by different strains, must incorporate trends from as many stages of the fermentation as possible. Thus, even if the second half of the fermentation is the most critical, it is not appropriate to ignore overall fermentation performance up to this point. The method suggested from findings of the present study takes into account all these considerations.

The analysis of the fermentation performance of the 20 strains revealed differences between some strains to be as much as twofold, which equates to about 3 days under our conditions. Such a difference is likely to have dramatic impacts on winery throughput and juice processing, particularly at the height of vintage, and no doubt forms an important criterion in the selection of strains by winemakers. In terms of the profiles of sugar utilisation, some common trends were seen. Thus, after an initial lag, sugar utilisation increased markedly, before this phase was followed by one of a slowing of utilisation. In addition an ordered utilisation was evident such that glucose was removed more rapidly than fructose. Similar reports of glucophilicity in industrial strains have been made by several authors [18, 23, 24, 36, 39]. Whilst the fermentation of a fructose medium was slower compared with an equivalent amount of mixed sugars, as reported recently [18], it is interesting that the relative strain performance in the former was not an effective predictor of performance in the mixed sugar condition.

A recent study of the ability of yeast to grow in a fructose-only medium [1] compared the area under growth curves to estimate preference or tolerance of different *Saccharomyces* yeast for fructose. This method was suggested as a possible tool for initial screening of yeast. However, with the caveat that our study examined sugar utilisation rather than growth, the mismatch we observed between a glucose and fructose mixture and that of fructose alone ($R^2 = 0.083$) suggests it unlikely that a fructose-only medium will be useful to screen for performance in an extended mixed sugar fermentation. The presence of glucose is highly influential on fructose metabolism [14, 18, 19, 21, 27, 28, 32, 37, 38]. At this point it is not possible to state how glucose influenced fructose consumption. The effect may be elicited at the level of the membrane (transport and sensing) or phosphorylation during the first steps of glycolysis, through a higher K_m for fructose compared with glucose. Thus, further work is necessary to better explain the complex interaction between yeast and the simultaneous presence of two or more sugars. For the moment, however, it is possible to affirm that this phenomenon is highly strain dependent and that fermentation performance in presence or absence of glucose can vary considerably between strains.

In attempting to define fermentation curves of different yeast strains in a mixed sugar medium, others have developed equations to fit fermentation profiles [39]. We chose not to do this, given the high frequency of sampling and good agreement between replicates in our study. Instead, we used the composite trapezoid rule to determine the AUC describing the fermentation of each sugar in either medium.

Table 3 One-way ANOVA with Tukey's multiple-comparison post-test analysis of 19 commercial yeast strains (and average of all strains) in terms of their ratio of the area under the curve of glucose utilisation and fructose utilisation from media containing both sugars at 115 g/l (as reported in Table 2)

Strain	S6U	EC1118	CRU-BLANC	QA 23	AWRI 1503	SYRAH	VIN 13	V1116	UVAFERM 43	UCD 522	AWRI 796	AWRI 350	NT 202	W 27	BM 45	PRIMEUR	D 254	BORDEAUX RED	ALL
FERMICHAMP	–	–	•	•	••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••
S6U		–	–	–	–	–	–	–	–	–	•	•	••	••	••	••	•••	•••	–
EC1118			–	–	–	–	–	–	–	–	–	•	••	••	••	••	•••	•••	–
CRU-BLANC				–	–	–	–	–	–	–	–	–	•	•	••	••	••	•••	–
QA 23					–	–	–	–	–	–	–	–	•	•	•	•	••	•••	–
AWRI 1503						–	–	–	–	–	–	–	–	–	–	–	•	•••	–
SYRAH							–	–	–	–	–	–	–	–	–	–	–	•••	–
VIN 13								–	–	–	–	–	–	–	–	–	–	•••	–
V1116									–	–	–	–	–	–	–	–	–	•••	–
UVAFERM 43										–	–	–	–	–	–	–	–	••	–
UCD 522											–	–	–	–	–	–	–	••	–
AWRI 796												–	–	–	–	–	–	••	–
AWRI 350													–	–	–	–	–	••	–
NT 202														–	–	–	–	•	–
W 27															–	–	–	–	–
BM 45																–	–	–	–
PRIMEUR																	–	–	–
D 254																		–	–
BORDEAUX RED																			•••

Significant differences were calculated at p value <0.05 (•), 0.01 (••) or 0.001 (•••). Non-significant differences are represented by "–"

The result is a bi-dimensional measurement of fermentation, which relates residual sugar concentration and duration of fermentation. This would appear to be the most inclusive approach to defining fermentation performance used to date. The subsequent comparison of areas under each of the fructose and glucose curves therefore provides an overall indication of the sugar affinity of each strain, taking into account all stages of fermentation. Importantly, the ratio of values derived using this approach also provides a convenient means for normalising data for strains that require different times to complete fermentations.

Other approaches to describe different utilisation rates of glucose and fructose whilst normalising data for different fermentation durations have been described. Berthels et al. [2] calculated the ratio between glucose and fructose at four points in a fermentation when 20%, 30%, 40% and 50% of the glucose had been consumed. Over this part of the fermentation they observed a linear increase in the glucose:fructose discrepancy. Moreover they were able to sort strains according to the slope of the increase in glucose:fructose discrepancy. The reason for selecting four specific residual glucose concentrations as the points for comparing all strains is not made clear, but presumably other points within this window would suffice. If not, following such a prescriptive approach would require a possibly unmanageable high degree of frequent and real-time glucose quantitation.

As an alternative to such real-time analysis, Guillaume et al. [15] plotted fermentation according to g/l of residual sugar normalised against g/l of CO_2 released, the latter being easily determine by weight loss measurements. With this approach they were able to rank two strains graphically based on their different pattern of fructose and glucose utilisation and thereby differentiate the strains. A shortcoming of the work arises from the fact that the curves were mathematically fitted and therefore clearly approximations. In a similar approach Dumont et al. [11] introduced a fructophilic index as a criterion by which to describe the abilities of yeast to consume fructose compared with glucose. These workers focussed on the area between the fermentation curves for glucose versus fructose, in the latter two-thirds of the fermentation curve (also expressed as g/l of residual sugars versus g/l of CO_2 released). Accordingly, strains showing the lowest value, due to smallest area of differences between the glucose and fructose curves, were said to be fructophilic and were presumed to perform better in situations with high fructose concentrations. Finally, Tronchoni and colleagues [36] fitted sugar consumption curves with various mathematical equations and achieved R^2 values of 0.95 and higher. This enabled them to confidently estimate the time necessary for different strains to consume 50% and 100% of glucose and fructose, and, in turn, the residual fructose concentration at these points. Thus, ultimately the comparison between strains was at one or two time points and did not consider the characteristics of the fermentation beyond these.

From our results, it was encouraging to observe that FERMICHAMP showed the highest ratio between glucose and fructose AUC (0.76). This result agrees with the finding described by Guillaume et al. [15] regarding the exceptional ability of this strain to consume fructose. Similarly, Berthels et al. [2] described the discrepancy in glucose and fructose for several strains, including EC1118, VIN13 and BORDEAUX RED. Similar results for these three strains have been found in our study, where BOREAUX RED was the slowest fructose fermenter, VIN13 medium and EC1118 one of the fastest. These similarities with other studies increase the confidence of considering our glucose/fructose comparative approach as a valid method in describing relative sugar consumption profile during fermentation. Moreover, it was possible to rank 19 commercial wine strains according to their ability to consume fructose in relation to glucose and identify which strain was significantly different from the others in terms of fructophilicity (Table 3; Fig. 2). Therefore, FERMICHAMP and BORDEAUX RED are located at the opposite ends of the chart, and they are the only two strains significantly different from the mean. Other strains such as S6U and EC1118 are significantly different from most of the strains belonging to the less fructophilic group, while strains such as D254 and PRIMEUR were significantly different from most of the strains grouped as the most fructophilic.

In this study, the sampling frequency produced comprehensive fermentation curves and obviated the need for curve fitting and limitations arising out of this means of approximation. The post-fermentation determination of residual sugars reduced the analytical burden during the fermentation, and the calculation of the AUC is the most comprehensive encapsulation of all aspects of the fermentation performance of each strain. Finally, the latter benefit can be said to apply to the ratio of the areas of the glucose and fructose curves, whilst such ratios also have the advantage of normalising performance of strains with regard to fermentation duration.

Conclusions

The study proposes a novel approach (AUC) to determine fermentation performances in wine yeast strains, with particular attention to identifying a methodology to describe the fructophilicity of the strains. Although one of the goals of the study was to use a medium containing fructose as sole carbon source to predict relative preference for fructose by strains and to relate this to fermentation performance, the complexity of glucose and fructose utilisation

for individual strains remains too great. Further work is warranted to ascertain the significance of fructose utilising capability to overall fermentation behaviour and wine quality, and thereby to optimize strain development programs.

References

1. Arroyo-Lopez FN, Querol A, Barrio E (2009) Application of a substrate inhibition model to estimate the effect of fructose concentration on the growth of diverse *Saccharomyces cerevisiae* strains. J Ind Microbiol Biotechnol 36:663–669
2. Berthels NJ, Cordero Otero RR, Bauer FF, Thevelein JM, Pretorius IS (2004) Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res 4:683–689
3. Berthels NJ, Cordero Otero RR, Bauer FF, Pretorius IS, Thevelein JM (2008) Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast strains. Appl Microbiol Biotechnol 77:1083–1091
4. Bisson LF, Block DE (2002) Ethanol tolerance in *Saccharomyces*. Biodivers Biotechnol Wine Yeast, 85–98
5. Bisson LF, Fraenkel DG (1983) Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 80:1730–1734
6. Boehringe-Mannheim (1989) D-glucose/D-fructose. In: Methods of biochemical analysis and food analysis. Boehringer Mannheim, pp 50–55
7. Cavazza A, Poznanski E, Trioli G (2004) Restart of fermentation of simulated stuck wines by direct inoculation of active dry yeasts. Am J Enol Vitic 55:160–167
8. Ciriacy M, Reifenberger E (1997) Hexose transport. In: Zimmermann FK, Entian KD (eds) Yeast sugar metabolism. Technimic, Lancaster, pp 45–65
9. Diderich JA, Schepper M, van Hoek P, Luttik MA, van Dijken JP, Pronk JT, Klaassen P, Boelens HF, de Mattos MJ, van Dam K, Kruckeberg AL (1999) Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*. J Biol Chem 274:15350–15359
10. Diderich JA, Schuurmans JM, Van Gaalen MC, Kruckeberg AL, Van Dam K (2001) Functional analysis of the hexose transporter homologue HXT5 in *Saccharomyces cerevisiae*. Yeast 18:1515–1524
11. Dumont A, Raynal C, Raginel F, Ortiz-Julien A (2009) The ability of wine yeast to consume fructose. Aust NZ Grapegrow Winemak 543:52–56
12. Elbing K, Larsson C, Bill RM, Albers E, Snoep JL, Boles E, Hohmann S, Gustafsson L (2004) Role of hexose transport in control of glycolytic flux in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:5323–5330
13. Gafner J, Schütz M (1996) Impact of glucose-fructose-ratio on stuck fermentations: practical experiences to restart stuck fermentations. Wein-Wissenschaft Vitic Enol Sci 51:214–218
14. Gonçalves P, Rodrigues de Sousa H, Spencer-Martins I (2000) *FSY1*, a novel gene encoding a specific fructose/H+ symporter in the type strain of *Saccharomyces carlsbergensis*. J Bacteriol 182:5628–5630
15. Guillaume C, Delobel P, Sablayrolles JM, Blondin B (2007) Molecular basis of fructose utilization by the wine yeast *Saccharomyces cerevisiae*: a mutated *HXT3* allele enhances fructose fermentation. Appl Environ Microbiol 73:2432–2439
16. Henschke PA, Jiranek V (1993) Yeast—metabolism of nitrogen compound. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic, Chur, pp 77–164
17. Jiranek V, Langridge P, Henschke PA (1995) Amino-acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically-defined medium. Am J Enol Vitic 46:75–83
18. Júnior MM, Batistote M, Ernandes JR (2008) Glucose and fructose fermentation by wine yeasts in media containing structurally complex nitrogen sources. J Inst Brew 114:199–204
19. Karpel JE, Place WR, Bisson LF (2008) Analysis of the major hexose transporter genes in wine strains of *Saccharomyces cerevisiae*. Am J Enol Vitic 59:265–275
20. Lee CK (1987) The chemistry and biochemistry of the sweetness of sugars. Adv Carbohydr Chem Biochem 45:199–351
21. Luyten K, Riou C, Blondin B (2002) The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. Yeast 19:713–726
22. McBryde C, Gardner JM, de Barros Lopes M, Jiranek V (2006) Generation of novel wine yeast strains by adaptive evolution. Am J Enol Vitic 57:423–430
23. Meneses FJ, Henschke PA, Jiranek V (2002) A survey of industrial strains of *Saccharomyces cerevisiae* reveals numerous altered patterns of maltose and sucrose utilisation. J Inst Brew 108:310–321
24. Meneses FJ, Jiranek V (2002) Expression patterns of genes and enzymes involved in sugar catabolism in industrial *Saccharomyces cerevisiae* strains displaying novel fermentation characteristics. J Inst Brew 108:322–335
25. Ozcan S, Johnston M (1995) Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. Mol Cell Biol 15:1564–1572
26. Ozcan S, Johnston M (1999) Function and regulation of yeast hexose transporters. Microbiol Mol Biol Rev 63:554–569
27. Perez M, Luyten K, Michel R, Riou C, Blondin B (2005) Analysis of *Saccharomyces cerevisiae* hexose carrier expression during wine fermentation: both low- and high-affinity Hxt transporters are expressed. FEMS Yeast Res 5:351–361
28. Ramos J, Szkutnicka K, Cirillo VP (1988) Relationship between low- and high-affinity glucose transport systems of *Saccharomyces cerevisiae*. J Bacteriol 170:5375–5377
29. Reifenberger E, Freidel K, Ciriacy M (1995) Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. Mol Microbiol 16:157–167
30. Rolland F, Wanke V, Cauwenberg L, Ma PS, Boles E, Vanoni M, de Winde JH, Thevelein JM, Winderickx J (2001) The role of hexose transport and phosphorylation in cAMP signalling in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 1:33–45
31. Rolland F, Winderickx J, Thevelein JM (2001) Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem Sci 26:310–317
32. Salmon J-M (1989) Effects of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. Appl Environ Microbiol 55:953–958
33. Salmon JM, Vincent O, Mauricio JC, Bely M, Barre P (1993) Sugar-transport inhibition and apparent loss of activity in *Saccharomyces cerevisiae* as a major limiting factor of enological fermentations. Am J Enol Vitic 44:56–64
34. Serrano R, Delafuente G (1974) Regulatory properties of the constitutive hexose transport in *Saccharomyces cerevisiae*. Mol Cell Biochem 5:161–171
35. Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA (2010) The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J Appl Microbiol. (doi: 10.1111/j.1365-2672.2009.04657.x)
36. Tronchoni J, Gamero A, Arroyo-Lopez FN, Barrio E, Querol A (2009) Differences in the glucose and fructose consumption profiles in diverse *Saccharomyces* wine species and their hybrids during grape juice fermentation. Int J Food Microbiol 134:237–243

37. Varela C, Cardenas J, Melo F, Agosin E (2005) Quantitative analysis of wine yeast gene expression profiles under winemaking conditions. Yeast 22:369–383
38. Verwaal R, Paalman JW, Hogenkamp A, Verkleij AJ, Verrips CT, Boonstra J (2002) *HXT5* expression is determined by growth rates in *Saccharomyces cerevisiae*. Yeast 19:1029–1038
39. Wang D, Xu Y, Hu J, Zhao G (2004) Fermentation kinetics of different sugars by apple wine yeast *Saccharomyces cerevisiae*. J Inst Brew Distill 110:340–346