Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains

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Abstract

While unfermented grape must contains approximately equal amounts of the two hexoses glucose and fructose, wine producers worldwide often have to contend with high residual fructose levels (>2 g l\(^{-1}\)) that may account for undesirable sweetness in finished dry wine. Here, we investigate the fermentation kinetics of glucose and fructose and the influence of certain environmental parameters on hexose utilisation by wine yeast. Seventeen *Saccharomyces cerevisiae* strains, including commercial wine yeast strains, were evaluated in laboratory-scale wine fermentations using natural Colombard grape must that contained similar amounts of glucose and fructose (approximately 110 g l\(^{-1}\) each). All strains showed preference for glucose, but to varying degrees. The discrepancy between glucose and fructose utilisation increased during the course of fermentation in a strain-dependent manner. We ranked the *S. cerevisiae* strains according to their rate of increase in GF discrepancy and we showed that this rate of increase is not correlated with the fermentation capacity of the strains. We also investigated the effect of ethanol and nitrogen addition on hexose utilisation during wine fermentation in both natural and synthetic grape must. Addition of ethanol had a stronger inhibitory effect on fructose than on glucose utilisation. Supplementation of must with assimilable nitrogen stimulated fructose utilisation more than glucose utilisation. These results show that the discrepancy between glucose and fructose utilisation during fermentation is not a fixed parameter but is dependent on the inherent properties of the yeast strain and on the external conditions.

Keywords: Fructose utilisation; Fructophilic yeast; Glucophilic yeast; *Saccharomyces cerevisiae*; Wine yeast; Wine fermentation

1. Introduction

In wine fermentation, the two main soluble sugars present in grape must, glucose and fructose, are co-fermented to ethanol and carbon dioxide, as well as other minor but important metabolites. Grape must usually contains equal or very similar amounts of glucose and fructose [1]. *Saccharomyces cerevisiae* is known to display a preference for glucose. Although fructose is used concomitantly with glucose, the latter is depleted first from the medium, which gives rise to a discrepancy between the amount of glucose and fructose consumed during fermentation (hereafter referred to as GF discrepancy). As a consequence, residual sugar in fermented grape must usually contains more fructose than glucose. Since fructose is approximately twice as sweet as glucose [2], its presence as residual sugar has a much stronger effect on the final sweetness of wine [3] and residual fructose is therefore the main cause of undesirable sweetness in dry wines. High residual fructose also means a lower ethanol yield and a higher risk for microbial spoilage of the finished wine. Finally, it has been reported that stuck fermentations are frequently
characterised by an unusually high fructose-to-glucose ratio [4]. Whether a causal relation between problem fermentations and a high fructose-to-glucose ratio exists remains unclear.

In spite of the importance of fructose fermentation for wine production, few studies have addressed this subject. Many must-mimicking synthetic media often do not even contain fructose [5–8]. Furthermore, no systematic analysis or quantification of the preference for glucose of wine yeast strains has been carried out. Although S. cerevisiae in general appears to be glucophilic, other yeast species, such as Candida stellata and Zygosaccharomyces bailii, have a clear preference for fructose [9,10].

To the best of our knowledge, the effect of environmental conditions on the preference for glucose has also never been assessed. It is therefore not known whether the ethanol accumulated during wine fermentation, unavailability of nutrients, or other stress conditions affect glucose utilisation and fructose utilisation differently. Nitrogen levels in particular have a major influence on wine fermentation. Indeed, the assimilable-nitrogen content of grape juice is often rapidly depleted during fermentation. As a result, most of the wine fermentation occurs with metabolically active cells in stationary phase. Although legally restricted, assimilable nitrogen (i.e., diammonium phosphate) may be added in limited quantities during wine fermentation, e.g., to reduce the risk of stuck or sluggish fermentations [11]. The effect of nitrogen supplementation on glucose and fructose utilisation, however, is not known. Identification of S. cerevisiae strains with a small GF discrepancy, as well as a better understanding of the effect of important environmental parameters in wine fermentation on this discrepancy might help solve the problems associated with sluggish/stuck fermentation and high residual fructose levels in finished wines.

We evaluated the GF discrepancy of a set of wine yeast strains and found pronounced differences between the strains. In addition, we showed that ethanol addition and nitrogen supplementation differentially affect the GF discrepancy.

2. Materials and methods

2.1. Yeast strains and culture conditions

Sixteen S. cerevisiae strains and one S. bayanus strain were used in this study (Table 1). The S. cerevisiae strains included 13 commercial wine strains, two strains isolated from tequila production (kindly provided by CIATEJ, Mexico) and a mutant (VIN2000) of a widely used commercial wine yeast (VIN13). Yeast cells were propagated under laboratory conditions at 30 °C in YPD medium, which contained 1% yeast extract (Biolab, Midrand, South Africa), 2% peptone (Fluka, Germany) and 2% glucose (Sigma, Germany). Solid medium was supplemented with 2% agar (Biolab).

2.2. Fermentation media

Colombard must was kindly provided by KWV (Paarl, South Africa). SO2 (50 mg l−1) was added at crushing. After pectolytic treatment and settling in the cold, the must was racked, but no filtration was performed. The juice was stored at 0 °C until use. The initial sugar content of the juice was 110.86 g l−1 of glucose and 107.23 g l−1 of fructose, pH 3.49, and total acidity (which is the sum of the titratable fixed and volatile acids and is expressed in terms of tartaric acid) was 7.8 g l−1. For the nitrogen supplementation experiment, Colombard must was supplemented with 500 mg l−1 of diammonium phosphate (Saarchem, Krugersdorp, South Africa).

The composition of standard synthetic must MS300 [8] was slightly modified in that it contained both 100 g l−1 of glucose and 100 g l−1 of fructose. MS30 and MS600 media were prepared in the same way, except that the total nitrogen content was reduced to 30 mg l−1 and increased to 600 mg l−1, respectively, whereas MS300 contained 300 mg l−1 total nitrogen.

For the ethanol addition experiments, 96% ethanol was added to the initial must in order to establish a 40 g l−1 ethanol level prior to inoculation. After five days of fermentation, another 40 g l−1 of ethanol was added to the fermenting must.

Table 1
List of wine yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIN13, WE372, NT112 and VIN7</td>
<td>S. cerevisiae</td>
<td>Anchor Yeast, South Africa</td>
</tr>
<tr>
<td>N96</td>
<td>S. cerevisiae</td>
<td>Anchor Yeast, South Africa</td>
</tr>
<tr>
<td>Bordeaux Red</td>
<td>S. cerevisiae</td>
<td>Lallemand Inc., Montréal, Canada</td>
</tr>
<tr>
<td>EC1118</td>
<td>S. cerevisiae or bayanus?</td>
<td>Lallemand Inc., Montréal, Canada</td>
</tr>
<tr>
<td>Sc22, Sc041, S102, 103, S325 and 3035</td>
<td>S. cerevisiae</td>
<td>Springer, France</td>
</tr>
<tr>
<td>MG, GV4</td>
<td>S. cerevisiae</td>
<td>CIATEJ, Mexico</td>
</tr>
<tr>
<td>VIN2000</td>
<td>S. cerevisiae</td>
<td>VIN13 UV-mutant, IWBT Stellenbosch</td>
</tr>
<tr>
<td>VR44</td>
<td>S. bayanus</td>
<td>Springer, France</td>
</tr>
</tbody>
</table>
2.3. Fermentation conditions

All fermentations were carried out in regular 750 ml wine bottles provided with a bubbling CO$_2$ outlet and containing 600 ml of the medium. The fermentation temperature was approximately 20 °C and no stirring was performed during any stage of the fermentation. Inocula were prepared as follows: one colony from a fresh YPD plate was inoculated into 200 ml of YPD broth and grown at 30 °C until a cell density of approximately 10$^6$ cfu ml$^{-1}$ was reached. The cells were counted and an equal amount of cells per strain was resuspended in the same medium as used for the fermentation. Each bottle was then inoculated with 5 ml of this cell suspension, corresponding to a final cell density of 10$^6$ cfu ml$^{-1}$, unless otherwise stated. The bottles were weighed daily to assess the progress of fermentation. The maximal fermentation rate was the maximum slope obtained from the representation of CO$_2$ production versus fermentation day and expressed as the amount of CO$_2$ per day (g day$^{-1}$). Samples of 25 ml each were taken at the indicated time points and analysed, after degassing, for glucose, fructose and ethanol levels by a GrapeScan FT120 (Foss Electric, Hilleroed, Denmark), which makes use of Fourier-transformed infrared spectroscopy for these determinations. Fermentations were conducted in triplicate.

3. Results

3.1. Assessment of the GF discrepancy of 17 Saccharomyces strains

The fermentation trials in Colombard must showed that all yeast strains tested utilised glucose more rapidly than fructose, confirming the glucophilic character of Saccharomyces wine yeast strains. A typical example of a fermentation profile is shown in Fig. 1 for strain N96. Even though the process started with approximately equal amounts of the two sugars, the concomitant but slower utilisation of fructose led to a discrepancy between the glucose and fructose levels (GF discrepancy) during the entire course of the fermentation. Directly after inoculation, a significant and variable amount of hexoses was taken up by all the strains. This initial rapid uptake was followed by a period of relatively steady sugar consumption. Towards the end of fermentation, when glucose became more limiting, the GF discrepancy decreased. When fermentation finally ceased, fructose was always found at significantly higher concentrations than glucose.

The initial uptake of hexoses was highly variable among the studied wine yeasts. While every precaution was taken to standardise precultures, it appears likely that the observed variations between strains are at least in part dependent on the preculture. We therefore disregarded the GF discrepancy generated during the early phase of the fermentation and focused on the fermentation stages when consumption of both fructose and glucose proceeded steadily. This was the case when between 20% and 50% of the glucose in the medium was consumed since the GF discrepancy increased almost linearly for all wine strains assessed for this period (Fig. 2). We ranked the strains in order of the rate of increasing GF discrepancy per amount of glucose consumed (dGF discrepancy/dG) (Fig. 3), calculated from the slopes of the graphs represented in Fig. 2.

For most of the strains investigated the increase in GF discrepancy was very similar. Compared to this majority of strains, only three strains showed a higher increase in GF discrepancy (Sc041, N96 and Bordeaux Red) and three strains a smaller increase in GF discrepancy (VIN2000, 3035 and Sc103).

![Fig. 1. Fermentation profile of N96. Co-fermentation of glucose and fructose during oenological fermentation of Colombard grape must: glucose (■); fructose (□); ethanol (▲); and CO$_2$ (●).](image1)

![Fig. 2. Discrepancy between the amount of glucose and fructose consumed at time points, where 20%, 30%, 40% and 50% of the glucose was consumed during fermentation by four commercial wine yeast strains: N96 (■); Sc041 (●); VIN13 (▲); and VIN2000 (◆). The experimental values were relative to zero at 20% glucose consumed for each strain.](image2)
While the GF discrepancy increased linearly while fermentation proceeded over the investigated period (Fig. 2), the fermentation rate gradually decreased. We therefore investigated whether the increase in GF discrepancy showed a correlation with the fermentation performance of the strains. However, there was no correlation between the increase in GF discrepancy and the fermentation rate of the strains (Fig. 3).

We also assessed whether the initial inoculum size influences the GF discrepancy. Two VIN13 fermentations with an inoculum of 10^1 or 10^3 cfu ml\(^{-1}\), respectively, displayed GF discrepancies that did not differ by more than 1.6% at the same fermentation stage (results not shown). Similarly, three Bordeaux Red fermentations with an inoculum of 10^1, 10^2 or 10^3 cfu ml\(^{-1}\), respectively, did not differ by more than 14% in their GF discrepancies at the same fermentation stage (results not shown). Hence, large variations in inoculum size did not influence the GF discrepancy of these strains significantly.

### 3.2. Influence of ethanol addition on glucose and fructose utilisation

The glucose and fructose utilisation of four commercial wine yeasts (VIN13, Bordeaux Red, N96 and VR44) was measured in the control must and in a must to which 40 g l\(^{-1}\) of ethanol had been added (Fig. 4). The amount of sugar consumed during the first five days of fermentation was assessed. During that time, sugar utilisation was inhibited in the must with added ethanol. Five days after ethanol addition, the strains had consumed between ca. 5% and 20% less glucose and between ca. 4% and 14% less fructose than in the control must. In all cases, glucose utilisation was inhibited more than fructose utilisation, with the difference in inhibition ranging from approximately 37% to 65%.

After the first five days of fermentation, another 40 g l\(^{-1}\) of ethanol was added into the same must and sugar utilisation was monitored for a further nine days. During that time, the strains had consumed between ca. 20% and 64% less glucose and between ca. 52% and 100% less fructose than in the control must. In all cases, fructose utilisation was inhibited more than glucose utilisation, with the difference in inhibition ranging from approximately 56% to 196%. Hence, under high ethanol conditions, fructose utilisation was inhibited more than glucose utilisation. Similar results were obtained with these strains when fermented in MS300 synthetic must (data not shown). From these results it appears that ethanol affects the utilisation of glucose and fructose differently; i.e., fructose utilisation seems to be inhibited more severely under high ethanol conditions (both in natural and synthetic grape must fermentations) than glucose utilisation.

### 3.3. Influence of nitrogen supplementation on glucose and fructose utilisation

The effect of the concentration of assimilable nitrogen, an important variable in winemaking, on the utilisation of glucose and fructose was investigated. Sugar consumption was evaluated after five days of fermentation in nitrogen-supplemented and non-supplemented Colombard must with the strains VIN13, Bordeaux Red, N96 and VR44 (Fig. 5). Total sugar utilisation was enhanced in the nitrogen-supplemented must. During this five-day period, the strains had consumed between ca. 6% and 9% more glucose and between ca. 13% and...
During the steady part of fermentation in a very similar manner, similar amounts of glucose and fructose, increases in GF discrepancy between the strains with regard to their glucose and fructose utilisation were noticed. The GF discrepancy of wine yeast strains during fermentation of fructose always lags behind that of glucose. Hence, wine yeast strains appear to be generally glucophilic. However, important differences between the strains with regard to their glucose and fructose utilisation were noticed. The GF discrepancy, which is a typical phenomenon when glucophilic wine yeasts ferment grape must (that naturally contains similar amounts of glucose and fructose), increases during the steady part of fermentation in a very similar way for the majority of the wine strains. However, 17% more fructose in the nitrogen-supplemented must. Fructose utilisation was thus increased more than glucose utilisation, with the difference in increase ranging from approximately 51% to 137%. Similar results were obtained with these strains as well as with strains Sc22 and 3035 in nitrogen-poor MS30 versus MS300 medium (data not shown). The same tendencies were also observed with the strains VIN13 and N96 in MS300 versus the nitrogen-rich MS600 medium (data not shown). Similar results were obtained with strain VIN13 in a sluggish fermentation of nitrogen-poor MS30 medium that was supplemented with 500 mg l\(^{-1}\) of diammonium phosphate at a late stage of fermentation (data not shown). Thus, regardless of the type of medium (natural or synthetic) and the strain background, fructose utilisation was increased proportionally more than glucose utilisation.

### 4. Discussion

The slow fermentation of fructose appears to be a major cause of high residual sugar levels in finished wine and is often associated with sluggish and stuck wine fermentations. The results show that, for all wine yeast strains investigated, fermentation of fructose always lags behind that of glucose. Hence, wine yeast strains appear to be generally glucophilic. However, important differences between the strains with regard to their glucose and fructose utilisation were noticed. The GF discrepancy, which is a typical phenomenon when glucophilic wine yeasts ferment grape must (that naturally contains similar amounts of glucose and fructose), increases during the steady part of fermentation in a very similar way for the majority of the wine strains. However, VIN2000, 3035 and Sc103 showed a slower, and Sc041, N96 and Bordeaux Red a faster rate of increase in GF discrepancy.

The results show that appropriate evaluation of the increase in GF discrepancy of wine yeast strains during strain selection could help to solve, at least partially, the problems caused by the slower fermentation of fructose. Hence, we suggest that determination of the GF discrepancy of candidate wine yeast strains should be a standard procedure in strain evaluation and selection.

Although ethanol is the major product of the oenological fermentation of grape must, it is inhibitory to the cells that produce it and constitutes a major stress factor during fermentation [12]. Ethanol inhibition stems mainly from its protein-denaturing properties. By diffusing freely through the yeast plasma membrane, ethanol causes damage not only to membrane proteins and to the phospholipid bilayer, but also to intracellular enzymes and structures. This results in, amongst other changes, increased membrane permeability and passive proton flux into the cell [13]. Wine yeasts in particular seem to be extremely tolerant of high ethanol levels, up to 12% (v/v) and more [6], but the reason for this is not clear. We observed that high ethanol levels generally inhibit the utilisation of sugar, but with a differential effect on the utilisation of glucose and fructose. In the beginning of the fermentation, fructose utilisation was less inhibited than glucose utilisation in ethanol-supplemented must. However, in the presence of high ethanol levels, fructose utilisation was significantly more inhibited than glucose utilisation. The glucose utilisation system thus seems to be more robust under conditions of high ethanol stress. This supports the notion that reduced fructose fermentation might be a major cause of sluggish and stuck fermentations. The sensitivity of fructose fermentation to high ethanol levels likely contributes to the fact that residual sugar in wine fermentations mainly consists of fructose. It is far from obvious why fructose fermentation is more sensitive to high ethanol levels. It has been described that ethanol affects the capacity, but not the affinity of the transport system for glucose [14]. Glucose and fructose are transported by the same carriers in wine yeast and at present 20 different genes encoding hexose transport-related proteins have been identified. Seven of them are actively involved in hexose transport, with Hxt1p and Hxt3p being the most relevant in winemaking conditions. It has been assumed that this diversity of hexose transporters, each bearing individual characteristics and kinetics, enables the yeast to effectively deal with a wide range of sugar concentrations. The transport system can roughly be divided in a high-affinity and a low-affinity transport system, operating under low and high external sugar concentration, respectively. For the high-affinity uptake system, \(K_m\) was previously determined as 1.5 ± 0.25 mM for glucose and 6 ± 2 mM for fructose. For the
low-affinity uptake system, $K_m$ was determined as $20 \pm 8$ mM for glucose and $40 \pm 15$ mM for fructose [15]. The affinity of the transport system is higher for glucose than fructose. It is unknown whether ethanol differentially affects the affinity of the transport system for glucose and fructose. ethanol also favours a shift in tautomeric equilibrium from fructopyranose to fructofuranose [16], and this may further lower the affinity of the transporters for fructose.

It was also noticed that the discrepancy in glucose and fructose utilisation changed during the course of fermentation. Early in the fermentation, when sugar utilisation rates were at a maximum, the GF discrepancy remained relatively small. As the total sugar utilisation slowed down, fructose utilisation was slowed down more than glucose utilisation, causing the GF discrepancy to increase to a maximum value. For some strains this discrepancy remained relatively constant, while for other strains it dropped again in the last stage of the fermentation. The GF discrepancy thus varied during the fermentation, revealing a typical pattern that was time- and strain-dependent. The reason for the different behaviour of the strains in the later stages of the fermentation is unclear. One possible explanation is a different ethanol sensitivity of the strains, in particular of the system responsible for the discrepancy in glucose and fructose fermentation. Another possible explanation is a different rate of nitrogen utilisation by the yeast strains. We noticed that fructose utilisation was stimulated to a greater extent by nitrogen supplementation than glucose fermentation. Hence, a different rate of nitrogen utilisation might impact on the rate of fructose fermentation. Nitrogen supplementation is often used to stimulate sluggish or stuck fermentations and our finding that fructose utilisation is more strongly stimulated helps to explain the efficiency of this treatment.

The precise molecular cause of the GF discrepancy is unclear. We have shown that this parameter is not simply related to the fermentation capacity of the strains. A higher or lower fermentation rate is not associated with a corresponding change in the fermentation rate of fructose. The metabolic pathway of fructose fermentation is very similar to that of glucose. Even the transporters are shared although their affinity for glucose is higher than for fructose, but the $V_{\text{max}}$ with the two sugars is similar. Hence, the transport step is a first candidate for the cause of the discrepancy in glucose and fructose fermentation. After transport glucose is phosphorylated by glucokinase, hexokinase 1 and hexokinase 2, whereas fructose is only phosphorylated by the latter two enzymes [17]. The affinity of the hexokinases is higher for glucose. Hence, the phosphorylation step is a second candidate for the cause of the discrepancy in glucose and fructose fermentation. Fructose is a ketose sugar, nearly 30% of which is present in the furanose form in solution [16], whereas glucose is an aldose, nearly 99.9% of which is present in the pyranose form [18]. Since glucose and other sugars are transported in the pyranose rather than in the furanose form, the actual transport-competent concentration of fructose is below its total concentration [12]. Differences in physicochemical properties like these may explain the lower affinity for fructose of the transport system [19] and the hexokinases [20,21].

After phosphorylation, fructose-6-P readily enters glycolysis by conversion into fructose-1,6-bisphosphate, while glucose-6-P still has to be converted first into fructose-6-P by phosphogluco-isomerase (PGI). The cause of the GF discrepancy therefore appears to be located in the transport and/or phosphorylation steps of the fermentation pathway. However, other possibilities also exist. Yeast cells are known to possess glucose sensor proteins in the plasma membrane of which at least one, Gpr1, is known to have a different affinity for glucose and fructose [22]. It is not known whether yeast cells possess a specific fructose sensor. Another relevant process differentially affected by glucose and fructose is catabolite repression. In the presence of rapidly fermentable sugars like glucose and fructose, yeast cells down-regulate the expression of genes involved in respiration, gluconeogenesis and the metabolism of alternative carbon sources [23]. The maintenance of catabolite repression by glucose requires Hxk2, while fructose catabolite repression requires either Hxk1 or Hxk2 [24]. Fructose repression may thus be triggered by a somewhat different mechanism compared to glucose repression. These data indicate that the discrepancy in glucose/fructose fermentation might not only be due to one or more differences in kinetic characteristics of transporters and initial enzymes of the fermentation pathway for their action on glucose and fructose as substrates.

Assimilable nitrogen is an essential nutrient that is critical for fermentation efficiency and that generally becomes limiting during wine fermentation [11]. The depletion of the nitrogen source, in combination with the rapid turnover of sugar transporters in the stationary phase, is thought to be responsible for inactivation of the sugar transport system and subsequent reduction in the fermentation rate observed towards the end of fermentation [25]. Hence, assimilable nitrogen might stimulate fructose fermentation preferentially by countering the degradation of hexose carriers with a higher affinity for fructose. In the present study, an increase in overall sugar utilisation was observed in high-nitrogen must as well as after the addition of ammonium at a late stage of fermentation. Fructose utilisation was enhanced to a higher degree than glucose utilisation in all cases. Re-addition of nitrogen source to nitrogen-starved cells in the presence of a fermentable sugar is known to activate the fermentable-growth-medium-induced (FGM) pathway, resulting, for example, in rapid induction of the whole set of ribosomal protein genes, the mobilisation of
trehalose and the activation of enzymes, including phosphofructokinase 2 [26]. To what degree these cell responses might be responsible for the more efficient utilisation of fructose remains to be investigated.

In conclusion, we have characterised the GF discrepancy of a set of wine yeast strains during fermentation of Colombard grape must and of synthetic must. We found a strain- and time-dependent variation in the discrepancy between glucose and fructose utilisation and we demonstrated differential effects of ethanol and nitrogen levels, two important wine fermentations parameters, on glucose and fructose fermentation. These results set the stage for a characterisation of the cause of the GF discrepancy at the molecular level.

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