Comparison of the Glycolytic and Alcoholic Fermentation Pathways of *Hanseniaspora vineae* with *Saccharomyces cerevisiae* Wine Yeasts

María José Valera 1*, Eduardo Boido 1*, Eduardo Dellacassa 2 and Francisco Carrau 1*

1 Área de Enología y Biotecnología de Fermentaciones, Facultad de Química, Universidad de la República, 11800 Montevideo, Uruguay; mariajose_valera_martinez@hotmail.com (M.J.V.); eboido@fq.edu.uy (E.B.)

2 Laboratorio de Biotecnología de Aromas, Facultad de Química, Universidad de la República, 11800 Montevideo, Uruguay; edellac@fq.edu.uy

* Correspondence: fcarrau@fq.edu.uy

Received: 11 July 2020; Accepted: 29 July 2020; Published: 3 August 2020

Abstract: *Hanseniaspora* species can be isolated from grapes and grape musts, but after the initiation of spontaneous fermentation, they are displaced by *Saccharomyces cerevisiae*. *Hanseniaspora vineae* is particularly valuable since this species improves the flavour of wines and has an increased capacity to ferment relative to other apiculate yeasts. Genomic, transcriptomic, and metabolomic studies in *H. vineae* have enhanced our understanding of its potential utility within the wine industry. Here, we compared gene sequences of 12 glycolytic and fermentation pathway enzymes from five sequenced *Hanseniaspora* species and *S. cerevisiae* with the corresponding enzymes encoded within the two sequenced *H. vineae* genomes. Increased levels of protein similarity were observed for enzymes of *H. vineae* and *S. cerevisiae*, relative to the remaining *Hanseniaspora* species. Key differences between *H. vineae* and *H. uvarum* pyruvate kinase enzymes might explain observed differences in fermentative capacity. Further, the presence of eight putative alcohol dehydrogenases, invertase activity, and sulfite tolerance are distinctive characteristics of *H. vineae*, compared to other *Hanseniaspora* species. The definition of two clear technological groups within the *Hanseniaspora* genus is discussed within the slow and fast evolution concept framework previously discovered in these apiculate yeasts.

Keywords: glycolysis; yeast; pyruvate kinase; non-*Saccharomyces*; fermentation evolution clade

1. Introduction

One of the main characteristics of yeast affecting their oenological use is their capacity to ferment sugars. Non-*Saccharomyces* yeasts have traditionally been considered bad fermenters. For that reason, selected strains of *S. cerevisiae* have been used in the oenological industry to ensure that complete fermentation occurs [1,2]. Recently, however, wineries have been encouraged to apply new, non-*Saccharomyces* species in winemaking processes to provide distinguishable flavours within wines [3–6]. Non-*Saccharomyces* species have been used to produce different aromas and flavours, compared with *Saccharomyces* strains [7,8]. Therefore, many efforts have been made to identify non-conventional yeast strains for oenological purposes [4,8,9].

The selection of oenological yeasts is commonly accomplished by identifying species from raw material. Spontaneously fermented grape musts are the niche that is most commonly used to identify novel strains that are both capable of fermenting sugars and confer desirable flavours to wines [10,11]. *Hanseniaspora* is the most abundant genus on grapes and grape juices. Studies have shown that up to 75% of the yeast population during the early stages of fermentation is made up of *Hanseniaspora* species [12,13]. After the first 48–72 h of spontaneous fermentation, the percentage of
Hanseniaspora species present decreases and the S. cerevisiae strains correspondingly increase. However, some Hanseniaspora species have been detected throughout the fermentative process [14]. Researchers have maintained that observed changes in yeast populations during fermentation occur, at least in part, because Hanseniaspora species are sensitive to ethanol [15]. S. cerevisiae is able to produce high quantities of ethanol rapidly. Therefore, this species dominates the fermentation process until sugar is completely depleted. Recent studies have shown the effects of antimicrobial peptides secreted by S. cerevisiae throughout the fermentation [16,17] inhibiting the growth of non-Saccharomyces yeasts. Therefore, the reputation of Hanseniaspora species as poor fermenters may be due to the presence of other inhibitors and not directly related to their reduced capacity to ferment sugars.

Not all the Hanseniaspora strains have the same properties. Species of the genus produce different secondary metabolites and exhibit different fermentative behaviours. In fact, differences could even be detected between strains belonging to the same species [18,19]. H. vineae is an epiphyte yeast that is not easily isolated from fruit, a feature it shares with all the S. cerevisiae strains [20]. H. vineae can be isolated from samples after one or two days of spontaneous fermentation in wines and other fruit beverages such as cider [19]. This highlights the distinct behaviour of the species, compared with the majority of Hanseniaspora apiculate yeasts, which are commonly isolated from the skin of grapes or grapevine soil. The ability of strains identified as H. vineae to complete grape juice fermentation has been demonstrated via single inoculation [7]. Moreover, selected strains of H. vineae contribute positively to wine aromas by providing floral and honey notes, even when sequential inoculation with S. cerevisiae strains was performed [21]. Our assessment of H. vineae showed that levels of phenylpropanoid flavours compounds synthesized from grape must were elevated compared with other yeasts. In H. vineae, the presence of metabolic pathways that actively transform aromatic amino acids explains the elevated phenyl acetate ester and benzenoid derived compounds synthesis compared to other yeasts and these flavour compounds provide fruity and flowery aromas [21–23].

Although several phenotypic studies have been carried out throughout wine fermentation using non-Saccharomyces species, there is a lack of information regarding the genetic basis of observed characteristics in non-Saccharomyces strains [8]. Due to the development of next generation sequencing, genomes of Hanseniaspora species from wine have been recently sequenced [24–27]. Further work will be needed to determine which genes are responsible for each function. In previous studies, the aromatic profile of H. vineae was correlated with genomics and transcriptomics data [22]. However, genes involved in glycolysis and fermentative behaviour in the species remain unknown. In S. cerevisiae, genes necessary for fermentation have been reported using mutant analysis. All of these, were grouped in a "fermentome" [28]. The genome of H. guilliermondii has been recently analysed [27] and the presence and absence of genes involved in the glycolytic and fermentative pathways compared with S. cerevisiae and other Hanseniaspora species were reported. Moreover, the H. uvarum glycolytic pathway has been assessed in a study that revealed the catalytic potentials of enzymes involved in the route [29]. The authors showed that the main glycolytic enzyme of H. uvarum, pyruvate kinase, had a 15-fold lower enzymatic activity than that of the S. cerevisiae enzyme.

The aim of this work is to establish the differences and similarities between H. vineae, other Hanseniaspora species, and S. cerevisiae regarding glycolytic and fermentative behaviour. In the present study, a comparative analysis of the fermentative capacity of H. vineae was performed using genetic and transcriptomic data. Characterization of the glycolytic and fermentative potential of H. vineae will enhance our understanding about the mechanisms and the regulation of the fermentative process in a non-Saccharomyces yeast. Hanseniaspora genus studies might help reveal new signs of S. cerevisiae domestication mechanisms for wine production.

2. Materials and Methods

2.1. Yeast Strains

Yeast strains used for this study are listed in Table 1.
Table 1. Yeast strains used in this study.

| Species         | Strain       | Source                        | Use                                           |
|-----------------|--------------|-------------------------------|----------------------------------------------|
| H. vineae       | T02/19AF     | Fermenting Tannat grape must (Uruguay) | Genomic, transcriptomic, phenotypic analysis |
| H. vineae       | T02/05AF     | Fermenting Tannat grape must (Uruguay) | Genomic and phenotypic analysis              |
| H. osmophila    | AWRI3579     | Fermenting Chardonnay grape must (Australia) | Genomic and phenotypic analysis              |
| H. uvarum       | AWRI1280     | Fermenting Tannat grape must (Uruguay) | Phenotypic analysis                          |
| H. uvarum       | AWRI3580     | Fermenting Chardonnay grape (Australia) | Genomic analysis                             |
| H. opuntiae     | AWRI3578     | Fermenting Chardonnay grape (Australia) | Genomic analysis                             |
| H. calicicensis | NRRL Y-1626  | Soil (Denmark)                | Genomic analysis                             |
| H. guillermondi | UTAD222      | Grape must (Portugal)         | Genomic analysis                             |
| S. cerevisiae   | 2885c        | Laboratory strain             | Genomic analysis                             |
| S. cerevisiae   | ALG804       | Oenological yeast (Oenobrands®) | Phenotypic analysis                          |

2.2. Fermentation in Natural Grape Must

Chardonnay grape must containing 300 mg N/L and 200 g/L of sugars at pH 3.5 was treated with 200 mg/L dimethyldicarbonate to prevent microorganism growth. Pre-cultures of H. vineae T02/19AF, H. vineae T02/05AF, H. uvarum AWRI1280, H. osmophila AWRI3579, and S. cerevisiae ALG804 were isolated from the Chardonnay grape must and incubated at 25 °C for 12 h in a rotary shaker at 150 rpm. Then, 125-mL Erlenmeyer flasks closed with cotton plugs used to simulate microaerobic conditions were inoculated with 75 mL of must containing 1 × 10^5 cells/mL. Static batch fermentations were conducted at 20 °C to simulate winemaking conditions.

2.3. Growth Kinetics in Different Types of Media

Six types of growth media were prepared using yeast nitrogen base (YNB) (Difco, Detroit, MI, USA) as a sole nitrogen source (6.7 g/L). Media were supplemented with the following carbon sources: Glucose, fructose, sucrose, xylose, glycerol, and maltose (2% w/w). YNB that lacked a carbon source was used as a negative control.

Chardonnay must used in the fermentation analysis was also used to measure the growth kinetics of yeast strains tested. Moreover, synthetic media that mimicked must fermentations at pH adjusted to 3.5 and ethanol concentrations of either 5% or 10% were used (20 g/L glucose, 4 g/L tartaric acid; 0.134 g/L sodium acetate; 5 g/L glycerol; and 1.7 g/L YNB) (v/v). The carbohydrate fermentation capacity was tested using Durham tubes immersed in media to detect gas production. Each type of medium tested was inoculated to produce a final concentration of 1 × 10^6 cells/mL in a final volume of 8 mL performed in triplicate. Results were visually assessed after a 48 and 96 h static incubation period at 25 °C.

2.4. Fermentation Ability in Different Carbon Sources

The carbohydrate fermentation capacity was tested using Durham tubes immersed in media to detect gas production. Each type of medium tested was inoculated to produce a final concentration of 1 × 10^6 cells/mL in a final volume of 8 mL performed in triplicate. Results were visually assessed after a 48 and 96 h static incubation period at 28 °C.

2.5. Genomic Analysis

Genomic DNA was obtained from H. vineae cultures grown in a YPD medium at 30 °C using the Wizard Genomic DNA Purification Kit (Promega, NY, USA), according to the manufacturer’s instructions.

The Illumina Genome Analyzer Iix platform in paired end mode was used to perform genomic sequencing as described previously [22]. Gene prediction was carried out using Augustus [30] trained
with S. cerevisiae gene models. Peptide predictions were then annotated using BLASTp (cutoff for e-value $1^{-10}$) against S. cerevisiae proteins, obtained from the Saccharomyces Genome Database [31].

A dendrogram was constructed using the sequences of nine genes encoding components of pathways related to glycolysis and fermentation from the Hanseniaspora species and S. cerevisiae. The genes assessed were CDC19, FBA1, PGI1, PFK1, PFK2, HXK2, ENO1, PGK1, and PDC1. Schizosaccharomyces pombe was used as an external group. Neighbour joining and Kimura 2-parameter methods were carried out using the MEGA version 4 software [32,33].

### 2.6. Transcriptomic Analysis

Fermentations were performed in triplicate using chemically defined grape (CDG) must with a composition similar to that of natural grape juice, but devoid of grape precursors. Components of CDG must were defined as described in Carrau et al. [34], with modifications. Briefly, glucose and fructose were added in equimolar concentrations until a total sugar concentration of 200 g/L was reached. Vitamins and salts were added as previously described [35]. Yeast available nitrogen (YAN) content was adjusted to 100 mg N/L. Of this total, 50 mg N/L corresponded to amino acids and 50 mg N/L corresponded to diammonium phosphate (DAP) supplementation, as described previously [35]. The pH of the media was adjusted to 3.5 using HCl and a final concentration of 10 mg/L ergosterol was the only lipid provided.

Pre-cultures of H. vineae T02/19AF were prepared in a CDG medium and incubated 12 h in a rotary shaker at 150 rpm and 25 °C. The pre-cultures were subsequently used to inoculate fermentation reactions carried out in 250 mL Erlenmeyer flasks that were closed with cotton plugs to simulate microaerobic conditions. For all strains, fermentations were performed using 125 mL CDG and an inoculum to produce $1 \times 10^5$ cells/mL in the final medium. Static batch fermentations were conducted at 20 °C to simulate winemaking conditions.

Wine samples for transcriptomic analyses were taken during the fermentation process at day 1 (exponential growth), day 4 (end of exponential phase), and day 10 (stationary phase of fermentation). For transcriptomic studies, total RNA obtained from H. vineae isolated from three replicates sampled from three different fermentation stages (days 1, 4, and 10) were analysed independently. The nine samples were paired-end sequenced using Illumina MySeq. Trinity software was used to assemble raw reads from transcriptomic analyses and further statistical analyses were performed as specified by Giorello et al. [22].

### 2.7. Statistical Analysis

All the treatments were performed in triplicate and the statistical error was calculated as the standard deviation of all data analysed. To compare growth and fermentation kinetics, variance comparison was performed by the ANOVA test carried out with STATISTICA 7.0 software. Differences in the mean absorbance or weight loss were evaluated using the Tukey test.

### 3. Results and Discussion

#### 3.1. Fermentative Capacity of H. vineae in Different Media

Hanseniaspora species used a limited number of carbon sources, which may have been related to the reduced competitiveness of the species throughout fermentations [27]. Regarding growth in different carbon sources (Figure 1A), growth of all the Hanseniaspora strains tested on both glucose and fructose had kinetics similar to that of S. cerevisiae ALG804. The media supplemented with sucrose was fermented by S. cerevisiae in a similar manner as that of media containing simple hexose. H. uvarum AWRI1280, however, did not grow on media containing sucrose. H. vineae T02/05AF, H. vineae T02/19AF, and H. osmophila AWRI3579 were able to grow on and ferment sucrose to an extent. Invertase gene (SUC2) is present in the genome of H. vineae. SUC2 is highly expressed on day 4 of fermentation reactions, but not day 1 or 10. However, other invertase homologs were not observed in
the genomes of any other Hanseniaspora species except H. osmophila [25]. Recently, Steenwyk et al. [36] grouped H. vineae and H. osmophila within the slower-evolving lineage of Hanseniaspora. In this branch, the SUC2 gene is present. This is different in species of the fast evolving lineage including H. uvarum, H. opuntiae, H. valbyensis, and H. guilliermondii, which might have lost the gene as a result of rapid mutation rates [36]. The same fact was detected with another key gene that show Saccharomyces wine yeast adaptations. Increased sulfite tolerance conferred by SSU1 (Table 2) is present in H. vineae and H. osmophila and it is absent in the other Hanseniaspora species. The presence of SUC2 and SSU1 genes are indicators of adaptations to alcoholic fermentation in yeast [37].

Glycerol was not used as a unique carbon source for H. vineae in accordance with data reported by Albertin et al. [38]. However, HvGUT1 and HvGUT2 genes were present in the genomes of both H. vineae strains analysed. In addition, xylose was not used by the H. vineae strains as expected. A finding that was likely due to the lack of enzymes needed to carry out the xylose conversion. The group of genes were also determined to be absent in H. guilliermondii, H. uvarum, and H. opuntiae [27]. However, H. vineae T02/05AF and T02/19AF have the ability to grow weakly when maltose is provided as a sole carbon source, despite the fact that they were not able to ferment the sugar. The same behaviour was also observed for H. jakobsenii [36].

Figure 1. Capacity of Hanseniaspora vineae and Saccharomyces cerevisiae to grow and ferment under varied conditions. (A) Growth of H. vineae and S. cerevisiae (G) and the capacity of the species ferment (F) when six different carbon sources (2% w/w) were provided. Yeast nitrogen base (YNB) that lacked a carbohydrate was used as a negative control. Black filled circles indicate that full growth and fermentation were observed, grey circles indicate the moderate capacity of species to grow and ferment, and white circles indicate that the species was not able to grow or ferment. (B) Growth kinetics of Hanseniaspora spp. and S. cerevisiae on the Chardonnay grape juice measure as increased absorbance over a period of 48 h. (C) Fermentation kinetics of the three strains in the Chardonnay grape juice after 12 days are shown. Growth and fermentation experiments were performed using independent triplicate samples and error bars express standard deviation.
As expected, *Saccharomyces* was able to grow and ferment sugars faster than *Hanseniaspora* species and significant differences between the species occurred after 16 h. The growth kinetics of the three *Hanseniaspora* strains tested were similar on grape must (Figure 1B), however fermentation kinetics of *H. vineae* and *H. osmophila* revealed that these species consume sugars significantly faster than *H. uvarum* (Figure 1C).

3.2. Sugar Transport

The transport of sugars into the cytosol of cells is a key step of the glycolytic pathway. *S. cerevisiae* is able to detect extracellular nutrients and make metabolic adjustments that rapidly facilitate the use of extracellular compounds [39].

Of the multiple sensors described in *S. cerevisiae*, *H. vineae* possessed the following genes HvSNF3, HvGPA2, HvGPR1, and HvASC1, which were determined to be associated with the hexose sensing capacity of both T02/19AF and T02/05AF strains (Table 2). ScSNF3 encodes a low glucose sensor present in the plasma membrane that is involved in the regulation of glucose transport and also has the capacity to sense fructose and mannose in *S. cerevisiae*. Expression of the gene in *H. vineae* increases throughout fermentation (Figure 1). ScGPA2, ScGPR1, and ScASC1 are hexose sensors that have been reported to be necessary for fermentation and are part of the “fermentome” in *S. cerevisiae*. Deletion of the genes was previously reported to induce protracted fermentation [28]. HvGPA2 and HvGPR1 have similar expression patterns throughout the fermentation process. The genes are maximally expressed on day 4 and their expression levels decrease at day 10. On the other hand, HvASC1 is most highly expressed on the first day of fermentation and levels were drastically reduced both on day 4 and 10 relative to day 1 (Figure 2).

*S. cerevisiae* possesses 20 sequences putatively associated with the hexose transport [40]. *H. guilliermondii* UTAD222 possess 22 sugar transporters, and based on their DNA sequences, ten were predicted to be associated with the hexose transport, all of them were most similar to *HXT2* with *S. cerevisiae* and significant differences between the species occurred after 16 h. The growth kinetics of the three *Hanseniaspora* strains tested were similar on grape must (Figure 1B), however fermentation kinetics of *H. vineae* and *H. osmophila* revealed that these species consume sugars significantly faster than *H. uvarum* (Figure 1C).

### Table 2. Genes involved in sugar transport, glycolysis, and alcoholic fermentation from *S. cerevisiae* and *H. vineae*. Gene copy numbers are detailed in brackets.

| Sugar transport and sensors | S. cerevisiae | H. vineae |
|----------------------------|---------------|----------|
| HXT (x17); SNF3; RGT2; FPS1; GPR1; GUP1; GU1P2; STL1; JEN1; ASC1; ASC2; GPA2; HXK1; HXK2; PG1; PFK1; PFK2; FBP1A; TP1; TDH1; TDH2; TDH3; PGK1; GPM1; ENO1; ENO2; CDC19; PYK2 | HXT (x2); SNF3; GPR1; GUP1; STL1 (x2); JEN1; ASC1; GPA2; HXK2; PG1; PFK1; PFK2; FBP1A; TP1; TDH2; TDH3; PGK1; GPM1; ENO1; ENO2; CDC19 |
| Glycolysis | PDC1; PDC2; PDC5; PDC6; ADH (x8) | PDC1; ADH (x8) |
| Key genes of wine yeasts adaptations | SSU1; SCP1 (x2); SUC2; THI1; THI12; THI13; THI14; THI16; THI20; THI21; THI72; THI73; THI80; TPC1 | SSU1; SUC2; THI7; THI72; THI80; TPC1 |

*S. cerevisiae* and *H. vineae* were predicted to be associated with the hexose transport, all of them were most similar to *HXT2* with *S. cerevisiae* and significant differences between the species occurred after 16 h. The growth kinetics of the three *Hanseniaspora* strains tested were similar on grape must (Figure 1B), however fermentation kinetics of *H. vineae* and *H. osmophila* revealed that these species consume sugars significantly faster than *H. uvarum* (Figure 1C).
which is needed to initiate glycolysis when glucose is provided as a carbon source and inhibits

According to the transcriptomic analyses, just one of the copies identified was di

H. guilliermondii

pyruvate, and acetate so that they can be used as carbon sources in

S. cerevisiae,

proton symporter of the plasma membrane, which has been shown to be inactivated in response to

2020

3.3. Glycolytic Pathway in H. vineae Strains

activity, amino acid homology was higher compared to other species of this genus.

Sc

S. cerevisiae,

Hv

beginning of fermentation reactions (days 1 and 4) and decreased at day 10 (Figure 1). One copy of

Hv

throughout fermentation in

S. cerevisiae

glucose in

Hv

is present in the genome of both T02/05AF and T02/19AF strains that shared homology with a glycerol

HXT6

proton symporter of the plasma membrane, which has been shown to be inactivated in response to

HXT6

transporter that is very similar to Sc

HXT6

late stages of fermentation (Figure 2).

HXT1

transmembrane transporter that transports glucose, fructose, and mannose [42]. Tondoni et al. [43]

revealed that in

S. cerevisiae

of sorbitol and mannitol.

H. guilliermondii,

other

Hanseniaspora

species sequenced, such as

H. osmophila

Hanseniaspora

H. osmophila

species sequenced, such as

H. opuntiae

H. guilliermondii, H. uvarum, and H. valbyensis also possessed between two and four copies of the gene.

According to the transcriptomic analyses, just one of the copies identified was differentially expressed

throughout fermentation in H. vineae (Figure 2).

HvFPS1, a putative plasma membrane channel involved in glycerol and xylitol movement, is present in the genome of both T02/19AF and T02/05AF. Expression of the gene is elevated near the beginning of fermentation reactions (days 1 and 4) and decreased at day 10 (Figure 1). One copy of HvGUP1 was present in each strain analyzed as well as HvJEN1. Moreover, it was suggested that ScGUP1 participates in glycerol transport and ScJEN1 mediates the high-affinity uptake of lactate, pyruvate, and acetate so that they can be used as carbon sources in S. cerevisiae [46,47].

3.3. Glycolytic Pathway in H. vineae Strains

The first enzyme of the glycolysis pathway is a hexokinase (Figure 3). ScHXK2 phosphorylates

S. cerevisiae

this isoform is principally responsible for glucose activation, which is needed to initiate glycolysis when glucose is provided as a carbon source and inhibits

ScHXK1 [41]. However, in H. vineae, HvHXK2 was the only enzyme identified with putative hexokinase activity, amino acid homology was higher compared to other species of this genus.
Phosphofructokinase activity was determined to be the second-most important glycolytic enzyme. The enzyme determines fermentation capacity and is indispensable for anaerobic growth. In *S. cerevisiae*, the enzyme is composed of two alpha and beta subunits that are encoded by ScPFK1 and ScPFK2, respectively. *Hanseniaspora* strains possess sequences homologous to both ScPFK1 and ScPFK2 subunits and similar to *S. cerevisiae*, the subunits form a hetero-octameric complex [29]. Protein
sequences of both HvPFK1 and HvPFK2 were most similar to *S. cerevisiae* (76.78% and 79.24%) and *H. osmophila* (76.46% and 77.50%) relative to the other *Hanseniaspora* species assessed (Figure 4A). Phosphofructokinase only works in the forward direction and is not involved in gluconeogenesis. In fact, three activities are required for gluconeogenesis: Pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose-1,6-bisphosphatase. No genes encoding the key gluconeogenic enzymes have been identified in *H. vineae*, *H. guilliermondii*, *H. uvarum*, *H. osmophila*, or *H. valbyensis* [27]. This explains why *Hanseniaspora* species are not able to grow when non-carbohydrate precursors such as pyruvate, amino acids, or glycerol are provided as energy sources. This is different than *S. cerevisiae*, which is able to grow on a variety of carbon sources including ethanol and lactate [48].

**Figure 4.** Genes involved in glycolysis. (A) Dendrograms showing the genetic distances between predicted amino acid sequences of enzymes involved in glycolysis from seven *Hanseniaspora* species and the *Saccharomyces cerevisiae* S288c strain. Amino acid homology was calculated for each *Hanseniaspora* strain against *S. cerevisiae*. (B) Amino acid sequences that correspond to the binding domains of fructose...
1,6-bisphosphate inducer of pyruvate kinase in *S. cerevisiae*. ScCDC19 and ScPYK2 genes were compared with predicted sequences of CDC19 from *H. uvarum* and *H. vineae*. Amino acids corresponding to the region that differ from CDC19 and PYK2 are highlighted in green and the position of residues are marked with an asterisk (*). (C) A heatmap describing the expression levels of genes putatively determined to be involved in glycolytic pathways of *H. vineae* 1, 4, and 10 days after the initiation of fermentation. Green and red colours indicate high and low levels of expression, respectively. Data are shown in triplicate.

Predicted amino acid sequences of phosphoglucose isomerase (PGI1) from *H. vineae* and *H. osmophila* were 86% similar to that of *S. cerevisiae*. Predicted PGI1 amino acid sequences from *H. uvarum*, *H. valbyensis*, *H. guilliermondii*, and *H. opuntiae* were approximately 71% similar to *S. cerevisiae*. This tetrameric enzyme is involved in the interconversion of glucose-6-phosphate and fructose-6-phosphate. Phosphoglucose isomerase activity has also been associated with the regulation of the cell cycle and gluconeogenic events of sporulation in *S. cerevisiae* [49,50].

Two copies of the *S. cerevisiae* ScENO1 gene that encodes an enolase were identified in *H. osmophila*, while only one copy was identified in other sequenced *Hanseniaspora* species. This enzyme catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a tetramer that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate. Three unlinked genes, ScTDH1, ScTDH2, and ScTDH3, encode related, but not identical, polypeptides that form catalytically active homotetramers with different specific glyceraldehyde 3-phosphate dehydrogenase activities in *S. cerevisiae* [51,52]. In *H. vineae*, only TDH2 and TDH3 homologues have been identified, and both were differentially expressed throughout fermentation (Figure 4C).

*H. vineae* strains T02/19AF and T02/05AF possess HvGUT1 and HvGUT2 genes. Both ScGUT1 and ScGUT2 are associated with glycerol kinase activities in the cytoplasm and mitochondria, respectively. Glycerol degradation is a two-step process that is mediated by GUT1 and/or GUT2. Under aerobic conditions, *S. cerevisiae* is able to utilize glycerol as a sole carbon and energy source [53]. Both of the enzymes have homologs that have been identified in *H. vineae* and *H. osmophila*, other *Hanseniaspora* species such as *H. uvarum*, *H. guilliermondii*, and *H. opuntiae* lack homologous of these genes [27].

Several specific activities associated with glycolytic enzymes of *S. cerevisiae* and *H. uvarum* have high degrees of similarity, which highlights the general conservation of glycolytic pathways and the downstream reactions involved in ethanol production [29]. Pyruvate kinase is a key enzyme that catalyzes an irreversible step of the glycolytic pathway. The position of the enzyme at the branchpoint between fermentation and respiration makes it a key determinant energy metabolism [54]. Recent work revealed that the pyruvate kinase activity enhanced the capacity of *S. cerevisiae* to ferment sugars versus *H. uvarum* [29]. The predicted proteins, Cdc19p, of *H. vineae* and *H. osmophila* are more homologous to the corresponding Cdc19p of *S. cerevisiae* than those of *H. uvarum* and other *Hanseniaspora* species (Figure 4A). When residues of the catalytic domain of ScCdc19p [55] are compared with those of *H. uvarum* and *H. vineae*, only one amino acid difference was identified; Asp265 was substituted with Gly269 in *H. uvarum* and *H. vineae* (Figure 4B). However, in the binding site of the allosteric activator, fructose 1,6-bisphosphate, two amino acid differences between *H. uvarum* and *S. cerevisiae* and one between *H. vineae* and *S. cerevisiae* were identified. The two differences identified between *H. uvarum* and *S. cerevisiae* are at the same positions (Figure 4B) as those identified in the PYK2 gene of *S. cerevisiae*, a paralog of CDC19 that is characterized by its low pyruvate kinase activity compared with the pyruvate kinase protein encoded by CDC19 (formerly PYK1) [54].

Expression levels of 13 *H. vineae* genes involved in the glycolytic pathway mainly decreased from day 1 to day 4 of fermentation and were maintained throughout the stationary phase (Figure 4C). This finding is in agreement to previous observations in *S. cerevisiae* [43]. However, levels of HvTDH2 expression remained high at both days 1 and 4 and decreased expression levels were observed at day 10. Additionally, expression of HvGUT2 peaked at days 4 and 10, and increased expression levels of the gene were not detected at day 1. Finally, HvGPM2 was not expressed under the conditions assessed.
3.4. Alcoholic Fermentation in *H. vineae* Strains

The pyruvate decarboxylase activity plays a key role in the alcoholic fermentation pathway. Three different pyruvate decarboxylase isozymes have been identified in the genome of *S. cerevisiae*: ScPDC1, ScPDC5, and ScPDC6. The function of pyruvate decarboxylase is the degradation of pyruvate into acetaldehyde and carbon dioxide. The enzyme is responsible for transferring the final product of glycolysis (pyruvate) to ethanol production [56]. In *H. vineae*, no sequences homologous to ScPDC5 and ScPDC6 were found and HvPDC1 was the only pyruvate decarboxylase isozyme identified in the species. In *S. cerevisiae*, ScPDC1 was strongly expressed in fermenting cells. The enzyme is conserved among yeast, bacteria, and plants. It is regulated by glucose and ethanol concentrations and also by itself [57]. The active enzyme has a homotetrameric structure and the enzyme has two known cofactors: Thiamin diphosphate (ThDP) and Mg^{2+} [58–60]. In *H. vineae*, genes involved in thiamine biosynthesis have not been identified and a similar finding was also reported in *H. guilliermondii* [27] and most other *Hanseniaspora* species [36]. It has been suggested that this may contribute to the low alcoholic fermentative capacity of *Hanseniaspora* species, the phenotype has been shown to be related to the weak pyruvate kinase activity of *H. uvarum* [29]. *S. cerevisiae* genes associated with thiamine production are upregulated in the stationary phase of growth. Oenological strains with improved expression levels of the genes have corresponding elevated rates of fermentation [61]. This phenomenon may result from vitamin depletion that occurs after the exponential phase.

Alcohol dehydrogenases, which catalyse the conversion of acetaldehyde to ethanol are key fermentative enzymes. Many alcohol dehydrogenases have been identified in *S. cerevisiae* including ScADH1, ScADH2, ScADH3, ScADH4, ScADH6, and ScADH7. Many homologues of *S. cerevisiae* alcohol dehydrogenases have been found in the *H. vineae* genome. *H. vineae* has the same number of copies of the genes as *S. cerevisiae*. Eight alcohol dehydrogenase genes are present in *H. vineae* species, compared to six in *H. osmophila*, and four in other sequenced species of *Hanseniaspora* such us *H. uvarum*, *H. guilliermondii*, *H. valbyensis*, and *H. opuntiae*. This may explain the improved adaptation of *H. vineae* to alcohol fermentation relative to other *Hanseniaspora*. It is noteworthy that of the eight HvADH sequences found in the genome of *H. vineae*, at least three HvADH6 genes are encoded in tandem. Increased copies of the gene may be associated with increased fermentation capacity, indicating that the alcohol dehydrogenase activity might be a key feature of alcoholic fermentation adaptations [62]. *H. vineae* has an enhanced tolerance to ethanol (Figure 5B) versus *H. uvarum* and *H. osmophila*, which are unable to grow in media containing 10% ethanol.

*H. vineae* and *H. osmophila* genes encoding putative alcohol dehydrogenases were grouped in two main clusters that contained either ADH1, ADH2 and ADH3 or ADH6 and ADH7 (Figure 5A), this is in agreement with the two multigenic families reported by Giorello et al. [22]. The clusters were formed according to the clustal alignment of predicted protein sequences, however, regarding adscription by a single homology with *S. cerevisiae* ADHs in the databases [22] produced some discrepancies. Therefore, HvADH6 homologs from *S. vineae* and *H. osmophila* were removed from the HvADH6 and HvADH7 cluster. Moreover, the HvADH1 homologous sequence of *H. vineae* is grouped in the cluster of ScADH6 and ScADH7.

HvADH genes display different expression patterns (Figure 5C). Two of four paralogous copies of HvADH6 were not differentially expressed at the time points analysed. Expression of one copy of ADH6 significantly declined between days 1 and 4 of fermentation. In addition, the expression of one copy of ADH3 was elevated on day 4 relative to day 1 (Figure 5C). These behaviours are similar to those of aryl alcohol dehydrogenases that facilitate the production of increased levels of alcohol by *S. cerevisiae* [63]. Therefore, HvADHs may be important for reducing levels of fusel aldehydes by producing increased levels of alcohol in *H. vineae* [22].
Figure 5. Characteristics that facilitate fermentation. (A) Dendrogram depicting relationships between the predicted amino acid sequences of several putative ADH genes. Hanseniaspora vineae sequences are indicated in red. (B) Growth of Hanseniaspora species and Saccharomyces cerevisiae in synthetic wine containing 5% of ethanol (solid line) and 10% ethanol (dotted line) for 48 h. Error bars are not shown to enhance clarity. SD < 0.05 for all samples. (C) A heatmap depicting expression levels of genes putatively involved in glycolytic pathways in H. vineae after 1, 4, and 10 days of fermentation. Green and red colours indicate high and low levels of expression, respectively. Data are shown in triplicate.

3.5. Hanseniaspora Genus as an Evolution Model for Alcoholic Fermentation Adaptations

The glycolytic potential of two strains of H. vineae were analysed using genetic, transcriptomic, and phenotypic data. Results explained the good performance of the species with respect to fermenting wine [7,21]. Findings also showed that the H. vineae behaviour was similar to traditional S. cerevisiae strains used in winemaking. Due to the outstanding capacity of H. vineae to produce aromatic metabolites, it was necessary to compare the capacities of the H. vineae strains to produce ethanol with...
S. cerevisiae. The high degree of similarity between glycolytic and alcoholic fermentation enzymes of H. vineae and H. osmophila with S. cerevisiae showed that the two species should be classified as fermenters, while the remaining Hanseniaspora species assessed were adapted to the fruit niche and were correspondingly included in the fruit group. In our experience, H. vineae strains cannot be isolated from the fresh grape fruits [19]. A dendrogram of concatenated DNA sequences from seven glycolytic and fermentation genes (Figure 6) indicated the presence of two clades of Hanseniaspora species, similar to findings of Steenwyk et al. [36] determined using genes from the DNA repair processes present within the genus. Interestingly, the fruit and fermentation clades shown in Figure 6 were correlated with the slow and fast evolution lineages defined by these authors. Branches were in agreement with phylogenetic classifications that were based on ribosomal genes [19]. It might be interesting to use the group as an evolution model to determine the mechanism by which the fermentation group diverged separately from the fruit group [36], giving less species diversity probably due to slow evolution mechanisms. Further work will be needed to understand whether the process might be an example of domestication, as has been proposed for S. cerevisiae wine and beer strains [64].

Previous studies have compared the fermentation capacity of two species belonging to the fruit group: H. guillermondi and H. uvarum [27,29], and the work presented here is the first assessment of a member of the fermentation group of Hanseniaspora.

![Figure 6. Dendrogram of seven concatenated DNA sequences from Hanseniaspora species constructed using the neighbour-joining method. The robustness of branching is indicated by bootstrap values (%) calculated for 1000 subsets. The entries in brackets correspond to NCBI BioSample identifiers.](image)

4. Conclusions

The results suggest that H. vineae is clearly better adapted to the fermentation niche compared to what we named as the Hanseniaspora fruit clade. These results are in agreement with a separately evolution divergence between the two clades of the genus Hanseniaspora as was proposed previously. Phenotypic behavior of H. vineae growth, ethanol tolerance, and fermentation kinetics are in agreement with the genetic and transcriptomic data provided. The results obtained demonstrate that H. vineae and a genetically closely related species, H. osmophila, behave similarly. Homologies of glycolytic and alcoholic fermentation enzyme sequences of both species were compared to S. cerevisiae, and the similarities observed allowed the differentiation of H. uvarum from H. osmophila and H. vineae. High sequence homology in these latter two species was observed for key genes involved in glycolysis such as HXK2, which encodes hexokinase, PFK1/PFK2 subunits of phosphofructokinase, and CDC19 that encodes pyruvate kinase. This homology could explain the improved fermentative performance observed for H. vineae compared with other Hanseniaspora species. The elevated number of copies of ADH genes in H. vineae might be associated with increased ethanol tolerance in the species. The presence of active
genes typically related to wine fermentation capacities in *H. vineae* and *H. osmophila* such as sulfite tolerance (*SSU1*) and sucrose hydrolyzing invertase (*SUC2*) differentiate both species from the other sequenced species of the genus. Taken together, findings reported here support the characterization of the *Hanseniaspora* genus into two different groups that are adapted to two different niches, fruit and juice fermentation. These results have contributed to the improved characterization of the genus and furthermore might support the importance of it as a model for further studies related to the genetic and evolutionary phenomena of yeast domestication processes.

**Author Contributions:** M.J.V., E.B., E.D., and F.C. conceived the study and its design; M.J.V. and F.C. wrote the manuscript; M.J.V. performed laboratory experiments and data analysis; E.B. carried out statistical analysis. All authors read and approved the manuscript.

**Funding:** This research was funded by Agencia Nacional de Investigación e Innovación (ANII), Application of *Hanseniaspora vineae* Project ALI_2_2019_1_155314 with Lage y Cia-Lallemand, Uruguay.

**Acknowledgments:** We wish to thank our Universidad de la Republica for basic support of this work: CSIC Group Project 802 and Facultad de Quimica, Uruguay.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Rainieri, S.; Pretorius, I.S. Selection and improvement of wine yeasts. *Ann. Microbiol.* 2000, 50, 15–31.
2. Fleet, G.H. Wine yeasts for the future. *FEMS Yeast Res.* 2008, 8, 979–995. [CrossRef] [PubMed]
3. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 2014, 14, 215–237. [CrossRef] [PubMed]
4. Mas, A.; Guillamón, J.M.; Beltrán, G. Non-conventional Yeast in the Wine Industry. *FEMS Yeast Res.* 2016, 7, 1–2. [CrossRef]
5. Comitini, F.; Capece, A.; Ciani, M.; Romano, P. New insights on the use of wine yeasts. *Curr. Opin. Food Sci.* 2017, 13, 44–49. [CrossRef]
6. Carrau, F.; Gaggero, C.; Aguilar, P.S. Yeast diversity and native vigor for flavor phenotypes. *Trends Biotechnol.* 2015, 33, 148–154. [CrossRef] [PubMed]
7. Lleixà, J.; Manzano, M.; Mas, A.; Portillo, M.d.C. *Saccharomyces* and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions. *Front. Microbiol.* 2016, 7, 1959. [CrossRef]
8. Masneuf-Pomarède, I.; Bely, M.; Marullo, P.; Albertin, W. The genetics of non-conventional wine yeasts: Current knowledge and future challenges. *Front. Microbiol.* 2016, 6, 1563. [CrossRef]
9. Varela, C. The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Appl. Microbiol. Biotechnol.* 2016, 100, 9861–9874. [CrossRef]
10. Esteve-Zarzoso, B.;戈斯丁卡, A.; Bobet, R.; Uriburu, F.; Querol, A. Selection and molecular characterization of wine yeasts isolated from the “El Penedes” area (Spain). *Food Microbiol.* 2000, 17, 553–562. [CrossRef]
11. Degre, R. Selection and Commercial Cultivation of Wine Yeast and Bacteria. In *Wine Microbiology and Biotechnology*; Fleet, G.H., Ed.; Taylor and Francis: New York, NY, USA, 1993.
12. Romano, P.; Capece, A.; Jespersen, L. Taxonomic and ecological diversity of wine and beverage yeasts. In *Yeasts in Foods and Beverages*; Springer: Berlin/Heidelberg, Germany, 2006; pp. 13–53.
13. Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The Effect of Non-*Saccharomyces* Yeasts on Fermentation and Wine Quality. *S. Afr. J. Enol. Vitic.* 2003, 24, 55–62. [CrossRef]
14. Zott, K.; Miot-Sertier, C.; Claise, O.; Lonvaud-Funel, A.; Masneuf-Pomarède, I. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int. J. Food Microbiol.* 2008, 125, 197–203. [CrossRef] [PubMed]
15. Pina, C.; Santos, C.; Couto, J.A.; Hogg, T. Ethanol tolerance of five non-*Saccharomyces* wine yeasts in comparison with a strain of *Saccharomyces cerevisiae*—Influence of different culture conditions. *Food Microbiol.* 2004, 21, 439–447. [CrossRef]
16. Albergaria, H.; Francisco, D.; Gori, K.; Arneborg, N.; Gírio, F. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl. Microbiol. Biotechnol.* 2010, 86, 965–972. [CrossRef] [PubMed]
17. Wang, K.; Dang, W.; Xie, J.; Zhu, R.; Sun, M.; Jia, F.; Zhao, Y.; An, X.; Qiu, S.; Li, X.; et al. Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. Biochim. Biophys. Acta Biomembr. 2015, 1848, 2365–2373. [CrossRef] [PubMed]

18. Moreira, N.; Pina, C.; Mendes, F.; Couto, J.A.; Hogg, T.; Vasconcelos, I. Volatile compounds contribution of Hanseniaspora guilliermondii and Hanseniaspora uvarum during red wine vinifications. Food Control 2011, 22, 662–667. [CrossRef]

19. Martin, V.; Valera, M.J.; Medina, K.; Boido, E.; Carrau, F. Oenological impact of the Hanseniaspora/Kloeckera yeast genus on wines—A review. Fermentation 2018, 4, 76. [CrossRef] [PubMed]

20. Fleet, G.H. Yeast interactions and wine flavour. Int. J. Food Microbiol. 2003, 86, 11–22. [CrossRef] [PubMed]

21. Medina, K.; Boido, E.; Fariña, L.; Gioia, O.; Gomez, M.E.; Barquet, M.; Gaggero, C.; Dellacassa, E.; Carrau, F. Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with Hanseniaspora vinae. Food Chem. 2013, 141, 2513–2521. [CrossRef]

22. Giorello, F.; Valera, M.J.; Martin, V.; Parada, A.; Salzman, V.; Camesasca, L.; Fariña, L.; Boido, E.; Medina, K.; Dellacassa, E.; et al. Genomic and transcriptional basis of Hanseniaspora vinae’s impact on flavor diversity and wine quality. Appl. Environ. Microbiol. 2019, 75, 1–20. [CrossRef]

23. Valera, M.J.; Boido, E.; Ramos, J.C.; Manta, E.; Radi, R.; Dellacassa, E.; Carrau, F. The mandelate pathway, an alternative to the PAL pathway for the synthesis of benzenoids in yeast. Appl. Environ. Microbiol. 2020, 86, 701–720. [CrossRef] [PubMed]

24. Giorello, F.M.; Berna, L.; Greif, G.; Camesasca, L.; Salzman, V.; Medina, K.; Robello, C.; Gaggero, C.; Aguilar, P.S.; Carrau, F. Genome Sequence of the Native Apiculate Wine Yeast Hanseniaspora vinae Tl02/19AF. Genome Announc. 2014, 2, e00530-14. [CrossRef] [PubMed]

25. Sternes, P.R.; Lee, D.; Kutyna, D.R.; Borneman, A.R. Genome Sequences of Three Species of Hanseniaspora Isolated from Spontaneous Wine Fermentations. Genome Announc. 2016, 4, e01287-16. [CrossRef] [PubMed]

26. Riley, R.; Haridas, S.; Wolfe, K.H.; Lopes, M.R.; Hittinger, C.T.; Göker, M.; Salamov, A.A.; Wisecaver, J.H.; Long, T.M.; Calvey, C.H.; et al. Comparative genomics of biotechnologically important yeasts. Proc. Natl. Acad. Sci. USA 2016, 113, 9882–9887. [CrossRef]

27. Seixas, I.; Barbosa, C.; Mendes-Faia, A.; Gülüden, U.; Tenreiro, R.; Mendes-Ferreira, A.; Mira, N.P. Genome sequence of the non-conventional wine yeast Hanseniaspora uvarum UTAD222 unveils relevant traits of this species and of the Hanseniaspora genus in the context of wine fermentation. DNA Res. 2019, 26, 67–83. [CrossRef]

28. Walker, M.E.; Nguyen, T.D.; Liccioli, T.; Schmid, F.; Kalatzis, N.; Sundstrom, J.F.; Gardner, J.M.; Jiranek, V. Genome-wide identification of the Fermentome; genes required for successful and timely completion of wine-like fermentation by Saccharomyces cerevisiae. BMC Genom. 2014, 15, 552. [CrossRef]

29. Langenberg, A.; Bink, F.J.; Wolff, L.; Walter, S.; Grossmann, M.; Schmitz, H. Glycolytic Functions Are Conserved in the Genome of the Wine Yeast. Appl. Environ. Microbiol. 2017, 83, 1–20. [CrossRef]

30. Stanke, M.; Keller, O.; Gunduz, I.; Hayes, A.; Waack, S.; Morgenstern, B. AUGUSTUS: Ab initio prediction of alternative transcripts. Nucleic Acids Res. 2006, 34, W435–W439. [CrossRef]

31. Cherry, J.M.; Hong, E.L.; Amundsen, C.; Balakrishnan, R.; Binkley, G.; Chan, E.T.; Christie, K.R.; Costanzo, M.C.; Dwight, S.S.; Engel, S.R.; et al. Saccharomyces Genome Database: The genomics resource of budding yeast. Nucleic Acids Res. 2012, 40, D700–D706. [CrossRef]

32. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 1980, 16, 111–120. [CrossRef]

33. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 2007, 24, 1596–1599. [CrossRef] [PubMed]

34. Carrau, F.M.; Medina, K.; Boido, E.; Farina, L.; Gaggero, C.; Dellacassa, E.; Versini, G.; Henschke, P.A. De novo synthesis of monoterpene by Saccharomyces cerevisiae wine yeasts. FEMS Microbiol. Lett. 2005, 243, 107–115. [CrossRef] [PubMed]

35. Henschke, P.A.; Jiranek, V. Yeast: Metabolism of nitrogen compounds. In Wine Microbiology and Biotechnology; Taylor and Francis: New York, NY, USA, 1993; ISBN 0-415-27850-3.

36. Steenwyk, J.L.; Opulente, D.A.; Kominek, J.; Shen, X.X.; Zhou, X.; Labela, A.L.; Bradley, N.P.; Eichman, B.F.; Čadež, N.; Libkind, D.; et al. Extensive loss of cell-cycle and DNA repair genes in an ancient lineage of bipolar budding yeasts. PLoS Biol. 2019, 17, e3000255. [CrossRef] [PubMed]
37. Gallone, B.; Steensels, J.; Prahl, T.; Soriaga, L.; Saels, V.; Herrera-Malaver, B.; Merlevede, A.; Roncoroni, M.; Voordecker, K.; Miraglia, L.; et al. Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. *Cell* **2016**, *166*, 1397–1410.e16. [CrossRef]

38. König, S. Subunit structure, function and organisation of pyruvate decarboxylases from various organisms. *Front. Microbiol.* **2016**, *6*, 1569. [CrossRef]

39. Forsberg, H.; Ljungdahl, P.O. Sensors of extracellular nutrients in Saccharomyces cerevisiae. *Curr. Genet.* **2001**, *40*, 91–109. [CrossRef]

40. Jordan, P.; Choe, J.Y.; Boles, E.; Oreb, M. Hxt13, Hxt15, Hxt16 and Hxt17 from *Saccharomyces cerevisiae* represent a novel type of polyl transporters. *Sci. Rep.* **2016**, *6*, 23502. [CrossRef]

41. Rendina, A.; De La Cera, T.; Herrero, P.; Moreno, F. The hexokinase 2 protein regulates the expression of the GLK1, HXK1 and HXK2 genes of *Saccharomyces cerevisiae*. *Biochem. J.* **2001**, *355*, 625–631. [CrossRef]

42. Reifenberger, E.; Freidel, K.; Ciriacy, M. Identification of novel HXT genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. *Mol. Microbiol.* **1995**, *16*, 157–167. [CrossRef]

43. Tondini, F.; Lang, T.; Chen, L.; Herderich, M.; Jiranek, V. Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* **2019**, *294*, 42–49. [CrossRef]

44. Ozcan, S.; Johnston, M. Function and regulation of yeast hexose transporters. *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 554–569. [CrossRef] [PubMed]

45. Ferreira, C.; Van Voorst, F.; Martins, A.; Neves, L.; Oliveira, R.; Kielland-Brandt, M.C.; Lucas, C.; Brandt, A. A member of the sugar transporter family, Stl1p is the glycerol kinase by fructose-1,6-bisphosphate. *J. Biol. Chem.* **1985**, *260*, 15013–15018. [CrossRef]

46. Grauslund, M.; Rønnow, B. Carbon source-dependent transcriptional regulation of the mitochondrial glyceraldehyde-3-phosphate dehydrogenase genes. *Mol. Gen. Genet.* **1986**, *204*, 310–316. [CrossRef]

47. McAlister, L.; Holland, M.J. Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. *J. Biol. Chem.* **1985**, *260*, 15019–15027. [CrossRef]

48. McAlister, L.; Holland, M.J. Isolation and characterization of yeast strains carrying mutations in the glyceraldehyde-3-phosphate dehydrogenase genes. *J. Biol. Chem.* **1985**, *260*, 15013–15018. [CrossRef]

49. Grassl, M.; Remnow, B. Carbon source-dependent transcriptional regulation of the mitochondrial glycerol-3-phosphate dehydrogenase gene, *GPT2*, from *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **2000**, *46*, 1096–1100. [CrossRef]

50. Boles, E.; Hollenberg, C.P. The molecular genetics of hexose transport in yeasts. *FEMS Microbiol. Rev.* **1997**, *21*, 85–111. [CrossRef] [PubMed]

51. Jurica, M.S.; Mesecar, A.; Heath, P.J.; Shi, W.; Nowak, T.; Stoddard, B.L. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* **1998**, *6*, 195–210. [CrossRef]

52. Kellermann, E.; Seeboth, P.G.; Hollenberg, C.P. Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (PDC1) from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **1986**, *14*, 8963–8977. [CrossRef] [PubMed]

53. Hohmann, S.; Cederberg, H. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes PDC1 and PDC5. *Eur. J. Biochem.* **1990**, *188*, 615–621. [CrossRef]

54. König, S. Subunit structure, function and organisation of pyruvate decarboxylases from various organisms. *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1998**, *1385*, 271–286. [CrossRef]
59. Muller, E.H.; Richards, E.J.; Norbeck, J.; Byrne, K.L.; Karlsson, K.A.; Pretorius, G.H.J.; Meacock, P.A.; Blomberg, A.; Hohmann, S. Thiamine repression and pyruvate decarboxylase autoregulation independently control the expression of the \textit{Saccharomyces cerevisiae} \textit{PDC5} gene. \textit{FEBS Lett.} 1999, 449, 248–250. [CrossRef]

60. Eberhardt, I.; Cederberg, H.; Li, H.; König, S.; Jordan, F.; Hohmann, S. Autoregulation of yeast pyruvate decarboxylase gene expression requires the enzyme but not its catalytic activity. \textit{Eur. J. Biochem.} 1999, 262, 196–201. [CrossRef]

61. Bataillon, M.; Rico, A.; Sablayrolles, J.M.; Salmon, J.M.; Barre, P. Early thiamin assimilation by yeasts under enological conditions: Impact on alcoholic fermentation kinetics. \textit{J. Ferment. Bioeng.} 1996, 82, 145–150. [CrossRef]

62. Molina, A.M.; Swiegers, J.H.; Varela, C.; Pretorius, I.S.; Agosin, E. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. \textit{Appl. Microbiol. Biotechnol.} 2007, 77, 675–687. [CrossRef]

63. Rossouw, D.; Bauer, F.F. Comparing the transcriptomes of wine yeast strains: Toward understanding the interaction between environment and transcriptome during fermentation. \textit{Appl. Microbiol. Biotechnol.} 2009, 84, 937. [CrossRef]

64. Gonçalves, M.; Pontes, A.; Almeida, P.; Barbosa, R.; Serra, M.; Libkind, D.; Hutzler, M.; Gonçalves, P.; Sampaio, J.P. Distinct Domestication Trajectories in Top-Fermenting Beer Yeasts and Wine Yeasts. \textit{Curr. Biol.} 2016, 26, 2750–2761. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).