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# Gene expression of specific enological traits in wine fermentation

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## Abstract

**Background:** Wine fermentation is a dynamic process and yeast has a precise genic regulation system that allow them to coordinate the gene transcription levels. However, the fermentation stage at which there is a great variation in the genic expression associated to a particular enological trait is often unknown and there are no simple experimental approaches to define it.
**Results:** To identify the most adequate stage in which to evaluate the expression of the genes associated to specific enological traits we identified three stages of fermentation using the industrial strain EC1118. These stages, called early (0-16.4 hrs), middle (16.4-88.7 hrs) and late (88.7 hrs onwards), were characterized according to changes in the speed of $CO_2$ production. The greatest nitrogen consumption velocity (1.016 g/L h) was achieved in the early stage. The most significant changes in the consumption of sugar and ethanol production acceleration occurred in the middle stage, and the greatest velocity of ethanol production (0.043%/h) and fructose consumption (0.338 g/L h) occurred in the late stage. The expression profiles for a set of genes characteristic of these processes were clearly defined during the stages of fermentation: identifying a peak for the genes related to the consumption of nitrogen during the early stage, followed by an increase in the expression of genes related to the consumption of sugars in the middle stage. Finally, during the late stage there is a decrease in the expression of genes involved in the consumption of sugar, except for *HXT*3 the levels of which remain high.
**Conclusions:** The kinetic and transcriptional profiles of the enological traits under study are clearly distinct and each is associated to a particular stage of the fermentation. Our results confirm that $CO_2$ production is a simple parameter to estimate the stages of the fermentation. Therefore, using the kinetics of $CO_2$ loss it is possible to select the most adequate moment to study the expression of genes associated to the main enological traits: sugar consumption and production of nitrogen and ethanol.

**Keywords:** enological traits, gene expression, *Saccharomyces cerevisiae*, wine fermentation, wine yeast.

## INTRODUCTION

The study of genic expression profiles associated to traits of enological relevance is currently of central importance in wine making, and several works have analyzed these phenotypes using a high throughput approach. Thus, Rossignol et al. (2003) evaluated the changes in the transcription levels during fermentation and found that more than 2000 genes vary their expression in response to changes in the culture medium. Other transcriptional studies in wine yeast strains have been carried out to evaluate the effect of nitrogen availability (Marks et al. 2003; Mendes-Ferreira et al. 2004; Jimenez-Marti and Del Olmo, 2008), response to rehydration (Rossignol et al. 2003), biomass

production (Gómez-Pastor et al. 2010) and ethanol tolerance (Stanley et al. 2010), amongst others. All these studies have focused on obtaining a global knowledge of the yeast transcriptome during fermentation; however, the stage at which there is a greater variation in the genic expression associated to a specific phenotype is often unknown particularly in industrial yeasts. Furthermore, in these studies the enological traits are evaluated at the end of the fermentation, however, the greatest phenotypic variation and the expression of genes associated to these occur in the early stages and therefore to consider the moment of greatest difference in the genic expression is highly relevant for its evaluation. Hence, it is necessary to develop an experimental approach that allows a definition of the fermentation stages and their relationship to the transcriptional and phenotypic changes.

In this work, a simple method based on the monitoring of $CO_2$ loss was implemented to determine the most adequate stage to study genic transcription in wine fermentation. Within this context, we analyzed the gene expression profiles and the phenotypes related to the production of ethanol and the consumption of sugar and nitrogen in the commercial strain EC1118 under synthetic wine fermentation conditions. The genes analyzed for each enological trait of interest were selected because on a global scale their genic products represent the main metabolic processes associated to these phenotypes.

## MATERIALS AND METHODS

### Strains and fermentation conditions

The wine yeast strain EC1118 was used in this study (Lalvin, Zug, Switzerland). The fermentations were carried out in the synthetic must described by Rossignol et al. (2003) containing 125 g/L glucose, 125 g/L fructose and 300 mg/L of assimilable nitrogen. The fermentations were carried out in 50 mL Falcon tubes containing 40 mL of synthetic must at 25ºC for 15 days without shaking. The inoculum consisted of 1 x $10^6$ cells/mL obtained from a 16 hrs culture in the same must with shaking. $CO_2$ production was monitored by weighing the tubes to determine weight loss.

### Fermentation stages

The points where each fermentation stage ends were defined as the moments where a change in the acceleration of $CO_2$ loss occurred. These points were obtained from the second derivative graph of $CO_2$ loss in time.

### Enological parameters

The residual sugar and ethanol production were determined according to Nissen et al. (1997). The consumption of nitrogen as yeast assimilable nitrogen (YAN) was determined using the formaldehyde titration method described by Aerny (1996). The kinetic parameters of $CO_2$ release and ethanol production were adjusted to a sigmoid non-linear fit. The glucose and fructose consumption measurements and YAN uptake were adjusted to a four-parameter logistic equation and third order polynomial non-linear fit respectively. The adjustments were done using GraphPad Prism version 5.00 (San Diego California USA).

### RNA preparation and QPCR

RNA extraction, reverse transcription and QPCR experiments were done according to Contreras et al. (2012). Results were analyzed using the LightCycler 4.0 software (Roche, Germany) and quantification of relative gene expression was done using the mathematical method described by Livak and Schmittgen (2001) and normalized with the *ACT1* and *SCR1* genes following Vandesompele et al. (2002). The relative expression trend of the genes associated to each phenotype at 6 hrs of fermentation was obtained by averaging the relative genic expression means for each point. The error associated to each measurement was also considered. This trend was graphed with the software Graph Pad Prism using the LOWESS function (Chambers et al. 1983).

## RESULTS AND DISCUSSION

### Stages of alcoholic fermentation

The production of $CO_2$ is easy to evaluate and is a widely used criterion for the study of fermentations in much the same way as the production of biomass, but a lot less complex. Although the production of biomass follows a similar sigmoidal pattern to the formation of $CO_2$ during alcoholic fermentation, they do not occur simultaneously (Rossignol et al. 2003; Varela et al. 2004). Nevertheless, the loss of $CO_2$ is an indicator of the fermentative processes making it more adequate to estimate the degree of metabolic activity of the cell. To establish the stages of the fermentation, a non-linear adjustment was carried out using the kinetics of $CO_2$ loss. The changes in acceleration were calculated from the second derivative of this function (Figure 1a) thus defining the limits of each stage of fermentation. Hence, in the early stage between 0 and 16.4 hrs, the velocity of $CO_2$ loss was 0.17 g/L h, whilst maximum velocity was achieved in the middle stage of fermentation, between 16.4 and 88.7 hrs with an average of 0.86 g/L h. Finally, in the late stage from 88.7 hrs onwards this parameter decreased again to 0.2 g/L h. This result shows that the early stage is characterized by an almost null $CO_2$ release, suggesting that it could reflect the onset of fermentation driven by the lag phase of cell growth and the progressive saturation of the medium with $CO_2$ as described previously (El Haloui et al. 1989). The middle stage is characterized by the maximum velocity and maximum acceleration of $CO_2$ release, a process that has been correlated to exponential cell growth (El Haloui et al. 1989). Finally the late stage is characterized by a decrease in $CO_2$ release, reflecting the inhibition of the fermentative process (El Haloui et al. 1989). These criteria may be used in any alcoholic fermentation given that all the kinetics of $CO_2$ loss are similar (Rossignol et al. 2003, Marullo et al. 2006).

### Enological parameters

During the fermentation, the enological parameters, glucose, fructose and YAN consumption, as well as the production of ethanol were measured (Figure 1). Non-linear adjustments to the kinetics of each parameter were carried out to determine the velocity of change of each of these phenotypes in the fermentation stages previously defined. The maximum average velocity of nitrogen consumption was obtained in the early stage of fermentation (1.016 g/L h). On the other hand, the lowest velocity of glucose and fructose consumption was observed during this stage (0.09 and 0.085 g/L h, respectively). This is reflected in a low velocity of ethanol production that reached a value of 0.007%/h.

The average consumption velocity of both sugars increased during the middle stage of the fermentation, however, the average velocity of fructose consumption was lower than that of glucose (0.197 and 0.290 g/L h, respectively). The same tendency was observed with the average velocity of ethanol production which increased to 0.038%/h. On the other hand, the average velocity of YAN consumption decreased to 0.871 g/L h. These results suggest that this was the most active stage of the fermentation, where the greatest number of changes occurred in the traits analyzed.

In the late stage of the fermentation, the average velocity of nitrogen source consumption decreased (an average of 0.160 g/L h). In addition, the highest velocity of glucose consumption was observed in this stage, reaching an average of 0.329 g/L h, however, there was no mayor difference with respect to the velocity observed during middle stage. On the other hand, the velocity of fructose consumption varied significantly between these two stages, reaching a maximum only during the late stage (0.338 g/L h), which explains why the majority of the residual sugar detected corresponds to this hexose. Finally, the highest production velocity of ethanol was obtained in this stage reaching 0.043%/h.

### Gene expression during alcoholic fermentation

To evaluate the gene expression involved in the consumption of nitrogen we analyzed the genes *GAP1, MEP2*, *TAT1* and *GNP1* that correspond to the amino acids, ammonium, tryptophan and glutamine permeases, respectively (Figure 2b). These genes are involved in the transport of ammonium, glutamine and tryptophan that represent around 80% of the YAN consumed during alcoholic fermentation (Chiva et al. 2009). Furthermore, we analyzed the gene *URE2* that encodes for the main negative regulator of the nitrogen catabolic repression mechanism. The maximum expression of genes related to this phenotype was observed at 6 hrs, the first point analyzed in the early stage of the fermentation. Subsequently, the transcript levels decreased and only halfway through the middle stage did the expression values equal those observed at 6 hrs post inoculation. The increase in

expression of these genes in the middle stage would correspond to changes in expression of *GAP1* and *GNP1*. The assimilation of nitrogen is regulated by the NCR mechanism (Magasanik, 2003), which allows the yeast to select the best nitrogen source in the culture medium. In the presence of preferred nitrogen sources, the transcription of genes involved in the consumption and assimilation of less preferred nitrogen sources is suppressed (Magasanik, 1992; Ter Schure et al. 2000). Of the genes analyzed, only *URE2* increased its transcript levels during the middle stage of the fermentation. This gene encodes for the NCR effector system which keeps the transcriptional factor Gln3p in the cytoplasm which in turn activates the expression of genes related to the assimilation of less preferred nitrogen sources (Mitchell and Magasanik, 1984; Magasanik, 1992; Blinder et al. 1996). The increase in its transcript levels indicates that the yeast is in a state of catabolic repression by nitrogen, which is confirmed by the high levels of YAN in the medium during this stage and towards the end of the fermentation (Figure 1b). This suggests that measuring the velocity of nitrogen consumption and the expression of the genes involved must be carried out during the early stage of fermentation, since it is here where the most significant changes occur.

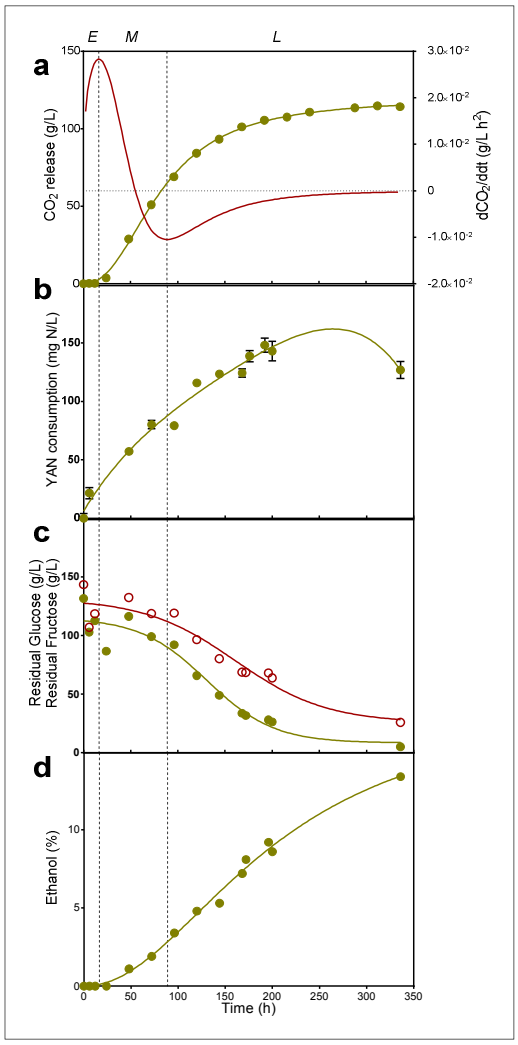


**Fig. 1 Enological profiles during alcoholic fermentation. *E, M, L* refers to the early, middle and late stages of the fermentation, respectively.** The dotted line shows where the middle stage starts and ends. Stages were determined as described in Materials and Methods. (a) $CO_2$ release; (b) YAN consumption; (c) sugar consumption (fructose and glucose); and (d) ethanol production during synthetic must fermentation. Each graph shows the experimental data (dots) and the mathematical fit (solid line).

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On the other hand, to analyze the expression of genes related to the consumption of carbon sources we selected the genes *HXK1*, *HXK2, HXT1* and *HXT3* that encode for hexokinases 1 and 2 and the low affinity hexose transporters 1 and 3, respectively. Furthermore, the transcriptional factors *CAT8* and *HAP4* and the glycolytic genes *CDC19* and *PGK1* which encode for pyruvate kinase and 3-phosphoglycerate kinase respectively were also analyzed. These genes showed a progressive increase in the levels of transcripts during the early stage and a maximum expression during the middle stage (Figure 2c), which correlates with the average consumption velocities of glucose and fructose. Finally, during the late stage of fermentation the levels of these gene transcripts decreased continuously.

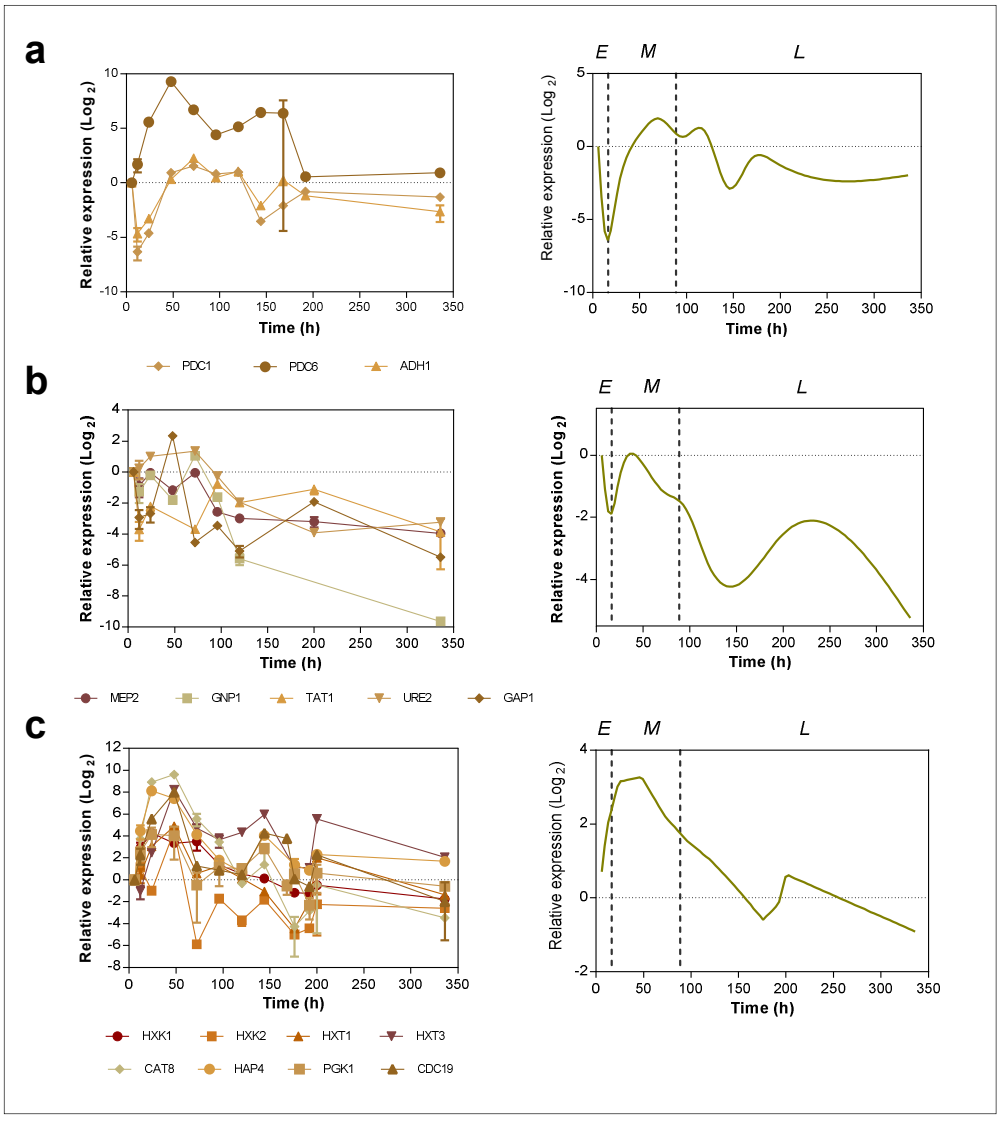


**Fig. 2 Gene expression profiles during alcoholic fermentation.** Changes in gene expression for ethanol production (a) nitrogen consumption (b) and sugar consumption (c) are expressed relative to the expression at 6 hrs from the beginning of fermentation. The primers used for gene expression analysis were obtained from the following references: Ethanol production, Dueñas-Sánchez et al. (2010). Nitrogen consumption, Chiva et al. (2009) (*GAP1* and *MEP2*) and Ma and Liu (2010) (*GNP1* and *TAT1*). Sugar consumption, Dueñas-Sánchez et al. (2010) (*HXK1*, *HKX2*, *CDC19* and *PGK1*), Vaudano et al. (2010) (*GAP4*), Biddick et al. (2008) (*HXT1* and *HXT3*); and Livak and Schmittgen (2001) (*CAT8*). Primers for URE2 were designed in this work (Fw: CCC GAA TTT GTG TCT GTG AA; Rv: TGC GTT GAT TTG TGA TTG GT). The tendency of the relative expression profiles for all genes through the different stages of fermentation is depicted at the right of each graph.

The highest velocity of $CO_2$ loss occurred during the middle stage of the fermentation which is consistent with an increase in biomass during this stage. The expression of the genes related to the consumption of sugars also increased and they all showed a similar expression profile (Figure 2c). This phenomenon precedes the point of maximum velocity in the consumption of glucose (130 hrs) and fructose (161 hrs) that occurred during the late stage of the fermentation. This may be explained by the time lag that exists between the expression of a gene and the effects of its genic product (Belle et al. 2006; Siwiak and Zielenkiewicz, 2010). However, genes *HXT1* and *HXT3* that encode for low affinity permeases (Reifenberger et al. 1997), showed different expression profiles, where gene *HXT1* was expressed in the first stage of the fermentation reaching a peak at 48 hrs. This is supported by Petit et al. (2000) who showed a positive regulation of this gene in response to the high concentrations of glucose and osmotic pressure present in the must initially. Subsequently, the expression of gene *HXT1* decreases rapidly and coincides with the increase in the velocity of glucose consumption. On the other hand, gene *HXT3* also showed a peak at 48 hrs of fermentation but its transcript levels remained high during the late stage, which could explain the consumption of fructose during the final stages of the fermentation as previously described (Pérez et al. 2005; Guillaume et al. 2007). These results show that the middle stage would be the most adequate to measure the expression of genes related to sugar consumption and evaluate changes in this enological trait.

To study the expression of genes related to ethanol production, we analyzed the genes *PDC1, PDC6* and *ADH1* that encode for the enzymes pyruvate decarboxylase 1 and 6 and alcohol dehydrogenase, respectively. These represent the most important isoforms of the enzymes involved in ethanol production (Figure 2a). During the early stage of the fermentation, the expression of the genes *ADH1* and *PDC1* decreased significantly with respect to the 6 hrs post-inoculation, a tendency which reverts during the middle stage where the maximum levels of transcripts were obtained for both genes. Finally, during the late stage of fermentation a sustained decrease in the transcript levels of these genes was observed. A different genic expression profile was observed for *PDC6* since its transcript levels increased continuously during the early stage of fermentation, reaching their maximum value halfway during the middle stage and maintaining these until the end. Observations have shown that under laboratory conditions the expression of gene *ADH1* varies in response to the concentration of glucose and ethanol, remaining high during the first stages of growth (De Smidt et al. 2012). However, under our assay conditions the transcript levels of *ADH1* were low in the initial stage of the fermentation. During the middle stage, when the velocity of glucose consumption increased and ethanol production began *ADH1* reached its maximum level of expression (between 12-72 hrs, Figure 2a). In agreement with this observation, the velocity of ethanol production during the middle stage increased approximately 6 times in comparison to that in the early stage.

A similar expression profile to *ADH1* was observed for the gene *PDC1*. This suggests a coordinated regulation of the two main genes involved in ethanol production and support the notion that halfway during the middle stage is the most adequate moment to carry out genic transcription studies associated to this phenotype.

Our results suggest that each enological phenotype and the associated gene expression must be evaluated in a particular stage of the fermentation, and these stages could be easily defined by the loss of $CO_2$.

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