Effect of deletion and overexpression of tryptophan metabolism genes on growth and fermentation capacity at low temperature in wine yeast

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Abstract

Low-temperature fermentations produce wines with greater aromatic complexity, but the success of these fermentations greatly depends on the adaptation of yeast cells to cold. Tryptophan has been previously reported to be a limiting amino acid during S. cerevisiae growth at low temperature. The objective of this study was to determine the influence of the tryptophan metabolism on growth and fermentation performance during low-temperature wine fermentation. To this end, we constructed the deletion mutants of the TRP1 and TAT2 genes in a derivative haploid of a commercial wine strain, and the TAT2 gene was overexpressed in the prototroph and auxotroph (∆trp1) backgrounds. Then we characterized growth and fermentation activity during wine fermentation at low and optimum temperatures. Our results partially support the role of this amino acid in cold yeast growth. Although deletion of TRP1 impaired amino acid uptake and the growth rate at low temperature in synthetic must, this growth impairment did not affect the fermentation rate. Deletion of TAT2 endorsed this strain with the highest nitrogen consumption capacity and the greatest fermentation activity at low temperature. Our results also evidenced reduced ammonium consumption in all the strains at low temperature.

Keywords: wine, cold, yeast, nitrogen, mutant, overexpressing strains, TAT2
30 **Introduction**

Many winemakers prefer low-temperature fermentation (10-15°C) for white and “rosé” wine production because it improves the characteristics of taste and aroma. This improved aroma profile can be attributed not only to the prevention of volatilization of primary aromas, but also to the increased synthesis of secondary aromas. Thus the final wine product has greater terpenes retention, a reduction in higher alcohols, and an increased proportion of ethyl and acetate esters in the total volatile compounds.\(^1\-^3\)

Another positive aspect is that low temperatures diminish the growth of acetic and lactic bacteria, which makes alcoholic fermentation control easier.\(^4\) However, low temperatures lower the growth rate and prolong the lag phase, which lead to longer fermentations and increase the risk of producing sluggish, stuck fermentations.\(^5\)

Therefore, the quality of wines produced at low temperature depends on the yeast’s ability to adapt to cold.

The nitrogen fraction in grape must consists of organic (amino acids) and inorganic (ammonium) nitrogen. *Saccharomyces cerevisiae* is able to regulate nitrogen uptake by the so-called nitrogen catabolite repression (NCR) mechanism.\(^6\) Recently, Crepin *et al.*\(^7\) determined the consumption kinetics of the amino acids and ammonium present in a synthetic grape must. These authors found that the assimilation sequence of the nitrogen compounds during fermentation is largely determined by the kinetic characteristics and regulation of the transporters of amino acids and ammonium. Most late consumed amino acids (i.e., arginine and alanine) are transported by permeases under NCR, except valine, tryptophan and tyrosine, which are transported by SPS-regulated permeases.\(^7\)

Quiros *et al.*\(^8\) mostly confirmed the order of consumption of the different nitrogen sources reported previously by Crepin *et al.*\(^7\) These authors also observed that the consumption of amino acids is also affected by both sugar concentration and
temperature. Beltran et al.\textsuperscript{9} showed that cells growing at low temperature had a less
effective NCR, which resulted in a higher uptake of two of these late consumed amino
acids, arginine and tryptophan, and that the consumption of glutamine, one of the earlier
consumed and more preferred amino acids, was lower. These authors\textsuperscript{9} also reported that
the consumption of ammonium in cells growing at low temperature significantly
diminished.

It has been reported that tryptophan uptake at low temperature is a rate-limiting step for
\textit{S. cerevisiae} growth.\textsuperscript{10} Broad-range permease (Gap1p), or specific amino acid
permeases Tat1p and Tat2p, are able to transport tryptophan into the cell. Tat2p
mediates the high-affinity uptake of tryptophan, and Tat1p mediates the low-affinity
uptake of this amino acid.\textsuperscript{11} Low temperature increases the rigidity of the plasma
membrane\textsuperscript{12}, which results in the slower lateral diffusion of membrane proteins, less
active membrane-associated enzymes, and a major reduction in membrane transport.\textsuperscript{13}
Membrane permeases are highly temperature-dependent because changes in temperature
can cause conformational changes in their structure.\textsuperscript{14} Sensitivity of tryptophan uptake
at low temperature has been related to a dramatic conformational change in Tat2p. Abe
and Horikoshi\textsuperscript{15} demonstrated that the overexpression of \textit{TAT2} in a laboratory \textit{S.
cerevisiae} strain confers yeast cells good capacity to grow at low temperature.
Moreover, enhancement of the gene expression for tryptophan biosynthesis or uptake
has also been related to ethanol stress tolerance.\textsuperscript{16} Recently in a metabolic comparison
between a wine strain of \textit{S. cerevisiae} and a cryotolerant strain of \textit{S. bayanus},\textsuperscript{17} we
detected more active tryptophan biosynthesis in the cryotolerant strain via the shikimate
pathway, which partially explains its improved growth at low temperature.
The aim of this study was to analyze the importance of tryptophan biosynthesis and
uptake in a context which mimics wine fermentation conditions. First we deleted the
TRP1 gene, which is involved in tryptophan biosynthesis, and the TAT2 gene, which is related to tryptophan uptake, in the genetic background of a derivative haploid of the commercial wine strain QA23. We also constructed strains which overexpressed the TAT2 gene in the background of the wine strain and in its auxotroph for tryptophan (Δtrp1). Then we analyzed the effect of deletions and overexpression on growth and fermentation activity during wine fermentations at low and optimum temperatures.

Materials and Methods

Construction of the mutant and overexpressing strains

The TAT2 and TRP1 genes were deleted on the derivative haploid of the commercial wine strain QA23 (Lallemand S.A., Canada) hoQA23. The primers used to construct the mutant and overexpressing strains are listed in Table 1. The TAT2 and TRP1 genes were deleted using the short flanking homology (SFH) method based on the KanMX4 and the NatMX4 deletion cassette, respectively, which provided geneticin (G418) and nourseothricin resistance. Given the high GC content of the NAT1 gene encoding nourseothricin-resistance, the PCR reaction mixture contained 5% of DMSO, as previously described. The deletion cassettes were used to transform the haploid hoQA23 strain by the lithium acetate procedure. After selecting the transformants in YPD with geneticin (G418) or nourseothricin (clonNAT), the correct insertion of the deletion cassettes was also verified by PCR using the primers upstream and downstream of the deleted region, combined with the primers of the KanMX4 or NatMX4 genes (Table 1).

The TAT2 gene was overexpressed by cloning it into the centromeric plasmid pGREG505 in strains hoQA23 and hoQA23 (Δtrp1), as described in Jansen et al. TAT2 was amplified from approximately 600 nucleotides upstream of the start codon to
400 nucleotides downstream of the stop codon in order to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94°C (2 min), followed by 30 cycles of 10 s at 94°C, 30 s at 52°C and 3 min at 72°C. The last cycle was followed by a final extension step of 10 min at 72°C. PCR fragments were generated with oligonucleotides containing short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp). The plasmid was linearized by SalI digestion and digested with AseI to avoid sticky ends and to make the recombination process easier. Derivative wine yeasts hoQA23 and hoQA23 (Δtrp1) were co-transformed with the digested pGREG505 plasmid, together with the PCR-amplified target gene that was flanked by recombination sequences homologous to the plasmid ends. This co-transformation promotes an in vivo homologous recombination between both fragments. This recombination process also deleted the GALI promoter of the plasmid (genes were cloned with their own promoters). Transformants were selected by geneticin resistance, which is encoded by the KanMX4 gene in the plasmid. Correct integration of the gene into the vector was verified by plasmid DNA isolation using a modified version of the protocol described by Robzyk and Kassir, and further PCR amplification was performed with the primers specified for sequences rec5 and rec2 (Table 1).

Growth media

Strains were cultured in the synthetic grape must (SM) (pH 3.3) described by Riou et al., but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors. The following were utilized: organic acids, malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L; mineral salts KH₂PO₄ 750 mg/L, K₂SO₄ 500 mg/L, MgSO₄ 250 mg/L, CaCl₂ 155 mg/L, NaCl 200 mg/L, MnSO₄ 4 mg/L, ZnSO₄
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4 mg/L, CuSO$_4$ 1 mg/L, KI 1 mg/L, CoCl$_2$ 0.4 mg/L, H$_3$BO$_3$ 1 mg/L and (NH$_4$)$_6$Mo$_7$O$_{24}$1 mg/L; vitamins myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotin 0.003 mg/L. The assimilable nitrogen source used was 300 mg N/L (120 mg N/L as ammonium and 180 mg N/L in the amino acid form). Tryptophan represented approximately 6.5% of the total assimilable organic nitrogen (12 mg N/L).

The population inoculated in the synthetic grape must came from an overnight culture in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30ºC. After counting microscopically, the appropriate dilution of the overnight culture was transferred to SM to achieve an initial cell concentration of 2 x 10$^6$ cells/mL.

*Gene expression analysis by real-time quantitative PCR*

The total RNA of 10$^8$ cell/mL was isolated from the different samples, as described by Sierkstra *et al.*, and was resuspended in 50 µL of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany), following the manufacturer’s instructions. RNA concentrations were determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and RNA quality was verified electrophoretically in 0.8% agarose gel. Solutions and equipment were treated to leave them RNase-free, as outlined in Sambrook *et al.*

Total RNA was reverse-transcribed with Superscript$^\text{TM}$ II RNase H$^\text{'}$ Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystems, USA). The reaction contained 0.5 µg of Oligo (dT)$_{12-18}$ primer (Invitrogen, USA) and 0.8 µg of total RNA as a template in a total reaction volume of 20 µL. Following the manufacturer’s guidelines, cDNA was synthesized at 42ºC for 50 min after denaturation at 70ºC for 10 min. Then the reaction was inactivated at 70ºC for 15 min.
The primers of the TAT2 gene were designed according to the Saccharomyces Genome Database. The primers of housekeeping gene ACT1 have been previously described by Beltran et al.\textsuperscript{25} (Table 1). Amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and the most precise quantification. Real-time quantitative PCR was performed using the LightCycler® 480 SYBR Green I Master (Roche, Germany). The SYBR PCR reactions contained 2.5µM of each PCR primer, 5 µL of cDNA and 10 µL of SYBR Green I Master (Roche, Germany) in a 20-µL reaction.

All the PCR reactions were mixed in a LightCycler® 480 Multiwell Plate 96 (Roche, Germany) and cycled in a LightCycler® 480 Instrument II, 96-well thermal cycler (Roche, Germany) under the following conditions: 95°C for 5 min, and 45 cycles at 95°C for 10 sec, at 60°C for 10 sec and 72°C 10 sec. Each sample had two controls that were run in the same PCR: no amplification control (sample without reverse transcriptase reaction) to avoid interference by contaminant genomic DNA, and no template control (sample without RNA template) to avoid interference by primer-dimer formation. All the samples were analyzed in triplicate with the LightCycler® 480 Software, version 1.5 (Roche, Germany), and the expression values were averaged. The gene expression levels of TAT2 in the overexpressing strains are shown as a relative value in comparison to their control strains, hoQA23-pGREG and pGREG (Δtrp1).

Housekeeping gene ACT1 was used as an endogenous reference gene to normalize input amounts. This endogenous ACT1 gene showed excellent uniformity in the expression levels of all the study samples.

\textit{Generation time (GT)}

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Germany) at 12°C and 28°C. Measurements were taken after pre-shaking the
microplate for 20 sec every 30 min for 3 days. At 12°C, the microplate had to be incubated outside the SPECTROstar spectrophotometer and was then moved inside to take measurements every 8 hours in the lag phase and every 3 hours in the exponential phase. Microplate wells were filled with 0.25 mL of SM medium to reach an initial OD of approximately 0.2 (inoculum level of 2 x 10⁶ CFU/mL). Uninoculated wells for each experimental series were also included in the microplate to determine, and to consequently subtract, the noise signal. All the experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering et al.²⁸:

\[ y = D \cdot \exp\{-\exp\left[\left(\frac{\mu_{\text{max}} \cdot e}{D}\right) \cdot (\lambda t) + 1\right]\} \]

where \( y = \ln(\text{OD}_t/\text{OD}_0) \), \( \text{OD}_0 \) is the initial OD, \( \text{OD}_t \) is the OD at time \( t \), \( D = \ln(\text{OD}_t/\text{OD}_0) \) is the asymptotic maximum, \( \mu_{\text{max}} \) is the maximum specific growth rate (h⁻¹), and \( \lambda \) is the lag phase period (h).²⁹ Generation time (GT) was calculated using the GT = ln2/\( \mu_{\text{max}} \) equation.

**Fermentations**

Fermentations were performed at 28°C and 12°C with continuous orbital shaking at 100 rpm, using 100-mL bottles filled with 60 mL of SM fitted with caps that enabled carbon dioxide to escape and samples to be removed. The population inoculated in each flask was 2 x 10⁶ cells/mL from an overnight culture in YPD. Fermentation was monitored by measuring media specific density with a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Fermentation was considered complete when density was below 0.998. Yeast cell growth was also determined by absorbance at 600 nm and by plating samples.
at the end of fermentation on YPD medium in serial decimal dilutions, and they were incubated at 28°C for 2 days.

**Nitrogen content analysis**

Samples were taken at 74 h and 240 h at 28°C and 12°C, respectively (around 1.020 of specific density), during fermentation. The individual amino acids present in the media were analyzed by OPA and FMOC derivatizations with the Agilent 1100 Series HPLC, as described in Beltran et al. Each amino acid concentration was calculated using external and internal standards, and was expressed as mg N/L. The Agilent ChemStation Plus software was used (Agilent Technologies, Germany).

**Statistical data processing**

All the experiments were repeated at least 3 times and data were reported as the mean value ± SD. Significant differences among the control strain, the mutant and the overexpressing strains were determined by t-tests (the SPSS 13 software package). The statistical level of significance was set at $P \leq 0.05$.

A principal component analysis (PCA) was done using the vegan package (the rda function) of the R v.2.15 statistical software (R Development Core Team, 2010).

**Results**

**Gene expression analysis of overexpressing strains by real-time quantitative PCR**

After constructing the overexpressing strains, we aimed to validate and quantify the gene expression in these strains. Samples were taken at time 0 h (before inoculation), 6 and 18 h after inoculation in the synthetic grape must (SM) at low temperature (12°C).
The relative expression values of *TAT2* are shown in Figure 1. The two overexpressing strains showed a significant overexpression of *TAT2* at time 0 h, which was 4-fold greater than the control for the pGREG *TAT2* strain and 27-fold greater than the control for the pGREG *TAT2* (∆*trp1*) strain. Moreover, the pGREG *TAT2* (∆*trp1*) strain also showed overexpression at 6 h and 18 h after inoculation (20- and 5.5-fold more than the control, respectively).

**Phenotype effect of the mutant and overexpressing strains**

**Determination of generation time (GT)**

In order to determine the effect of the *TRP1* and *TAT2* deletions on growth, and the overexpression of *TAT2*, in two different genetic backgrounds (tryptophan prototroph and auxotroph), the GT of the mutant and overexpressing strains at 12°C and 28°C in the SM were calculated (Table 2). No significant differences in the growth rate of the *TAT2* mutant and the overexpressing strains were found, and only the ∆*trp1* strain showed lower growth rate than its control strain, *hoQA23*, at both temperatures.

**Fermentation activity**

The fermentation activity of the mutant and overexpressing strains was estimated by calculating the time required to ferment 5% (T5), 50% (T50) and 100% (T100) of the sugars in the SM (Fig. 2). T5, T50 and T100 approximately matched the beginning (lag phase), middle (end of exponential phase) and end of fermentation, respectively. At the beginning of fermentation (T5), no differences were observed at low temperature. The ∆*tat2* strain displayed greater fermentation activity at low temperature, and required a considerably shorter time to consume 50% and 100% of the sugars (17 h and 60 h less, respectively) if compared to the fermentation of the control *hoQA23* strain at 12°C. Conversely this strain finished later than the control for the fermentation at 28°C.
pGREG TAT2 also finished earlier at 12°C if compared to its control (hoQA23-pGREG), but took longer to finish the fermentation at 28°C. pGREG TAT2 (Δtrp1) was able to finish fermentation at 28°C before its control pGREG (Δtrp1), but no differences were found at 12°C. These differences in fermentation activity cannot be ascribed to biomass production because non significant differences were observed in the viable population at the end of the fermentations among the different strains and temperatures, except for the Δtrp1 strain, which showed a larger population size at 28°C (Table 3).

Nitrogen consumption of mutant and overexpressing strains

Different nitrogen requirements in yeast fermenting were observed at both temperatures. At the same fermentation point (around 1.020 of specific density, which matches the end of the exponential phase), nitrogen consumption by yeast was generally lower at 12°C than at 28°C (Table 4). These differences between both temperatures were greater for ammonium consumption. Major differences in the amino acids present in the SM between the temperatures were observed in the tryptophan auxotroph strains (Δtrp1 and pGREG (Δtrp1)). Conversely mutations and overexpressions did not significantly modify the uptake of ammonium and amino acids at the same temperature. Only pGREG TAT2 (Δtrp1) at 28°C consumed more amino acids and ammonium than its control pGREG (Δtrp1).

In order to explore the effect of deletion/overexpression and temperature fermentation on ammonium and amino acid preferences, a PCA was carried out (Fig. 3). This PCA was performed on the seven strains using the residual concentration (mg N/L) of 20 amino acids and ammonium measured in the SM at the analyzed fermentation time point (1.020 of specific density) (Table S1). The first two components were retained and explained 86.7% of total variance. The first component (PC1) explained 68.8% of
variation, and was marked by a high positive component loading for histidine (+0.366) and high negative loadings for glutamine (-0.755) and ammonium (-0.509). The second component explained 17.9% of variation, and was marked by a high positive component loading for glutamine (+0.495) and a high negative loading for ammonium (-0.841). This result revealed the different nitrogen preferences at both temperatures. The most evident result was that control strain hoQA23 and its mutants consumed more histidine at the low temperature, and more glutamine and ammonium at the optimum temperature.

Discussion

Low temperature produces several effects on biochemical and physiological properties in yeast cells. Recently in their metabolomic study on wine fermentations at low temperature, López-Malo et al.\textsuperscript{17} reported that the main differences in the metabolic profiling of the \textit{S. cerevisiae} industrial strain QA23 growing at 12°C and 28°C were observed in the lipid and amino acid metabolisms. These differences in metabolites might be related to the previously reported different nitrogen consumption pattern at low temperature,\textsuperscript{8,9} or with the greater demand of some specific amino acids at low temperature, such as tryptophan.\textsuperscript{8,10} In this study, most strains also showed higher nitrogen consumption at the optimum temperature than at 12°C. As already reported by Beltran et al.\textsuperscript{9}, the main differences in nitrogen use at both temperatures are accounted for by ammonium consumption. This result supports the idea that there is a problem with the uptake or metabolization of one of the main nitrogen sources at low temperature.\textsuperscript{9} As previously reported\textsuperscript{31}, low temperature exerts strong nutritional stress, similarly to growth under nitrogen-limiting conditions. Regarding amino acid consumption, only the tryptophan auxotroph strains (\textit{Δtrp}1 and pGREG (\textit{Δtrp}1))
exhibited impaired amino acids uptake at low temperature, which was not affected at the optimum temperature. This lower amino acid consumption resulted in a growth defect in the ∆trp1 strain, which showed a longer GT at 12°C in comparison with the wild type, as previously reported.\textsuperscript{32} Nevertheless, we also observed impaired growth at the optimal temperature. It is important to consider that our experimental approach differs substantially from those previously reported using laboratory strains and growth conditions in standard laboratory media. We used an industrial wine strain to mimic wine fermentation conditions. Similar non fitting results were also obtained from the fermentation activity of this ∆trp1 strain. Some authors have revealed that the deletion mutants of tryptophan biosynthesis show ethanol-sensitive growth at the optimal temperature.\textsuperscript{16} In comparison with the control strain however, no significant differences were found with the ∆trp1 strain during the time required to complete fermentation.

The cold-sensitive phenotype of the ∆trp1 strain has been related with impaired tryptophan uptake. Abe and Horikoshi\textsuperscript{15} postulated that the increased rigidity of the plasma membrane at low temperature induces conformational changes in permease Tat2p and impairs tryptophan transport. In the present study, no differences were found in the growth rate of ∆tat2 at both temperatures if compared to hoQA23. Thus in this industrial strain, tryptophan uptake must be compensated by the action of other permeases, such as low-affinity tryptophan permease, Tat1p or the general amino acid permease Gap1p. Although it was not statistically significant, this strain consumed more amino acids and ammonium than its control strain hoQA23 at low temperature. However one unexpected result revealed that this strain also displayed better fermentation performance at low temperature. Although this direct correlation between TAT2 deletion and fermentation improvement at low temperature should be confirmed with other industrial strains, our working hypothesis is that the greater nitrogen uptake
of this strain produces a fermentation activity stimulus. Regulation of glycolytic enzymes by ammonium and amino acids uptake has been widely reported. Abe and Horikoshi demonstrated that the cells expressing Tat2p at high levels are endowed with the ability to grow under low-temperature conditions in a tryptophan auxotroph laboratory strain. However, the present study evidences no growth improvement with the overexpression of TAT2 in either the prototroph or the auxotroph background, and this result highlights the importance of the genetic background. As with the mutant strain, the overexpression of TAT2 also significantly reduced fermentation time.

Unexpectedly tryptophan uptake showed few differences between the different strains and temperature. This amino acid was practically consumed completely under all the fermentation conditions (Table S1). The PCA only revealed lower ammonium and glutamine consumption, as previously reported, and higher histidine consumption at low temperature, which has not been reported to date.

Conclusions

One conclusion drawn from this study is the need to test the phenotypes observed in the mutant laboratory strain in the genetic background of industrial strains under conditions that mimic the industrial process. Our experience has revealed that many of these phenotypes are not reproduced when transferred to more complex, robust and polyploid industrial strains. Tryptophan has been previously reported to be a limiting amino acid during S. cerevisiae growth at low temperature, and the results of the present study partially support this assessment. Deletion of TRPI, a key gene of tryptophan biosynthesis, impaired the growth rate at low temperature in synthetic must. However, it was also affected at 28°C. Thus the cold sensitivity of this strain in this genetic
background was not absolutely conclusive. Moreover this growth impairment did not affect the fermentation rate. A more evident phenotype in Δtrp1 was the lowest amino acid consumption noted at low temperature, which might explain its lower growth rate. Strangely enough, the overexpression of TAT2 in the Δtrp1 strain did not reveal that nitrogen consumption recovered sufficiently, whereas deletion of TAT2 (Δtat2) endorsed this strain with the highest nitrogen consumption capacity at low temperature. This strain underwent significant reduction during the time required to complete fermentation at low temperature. Although this result was unexpected, it may prove promising to meet the objective of obtaining industrial strains with improved fermentation capacity at low temperature through the selection of proper strains or the genetic modification of current industrial yeast.

Acknowledgments

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Literature cited


Figure legends

Figure 1. Relative expression of TAT2 during SM fermentation at 12ºC. Changes in gene expression in the overexpressing strains are shown as compared to their controls, hoQA23-pGREG and pGREG (Δtrp1), at the same fermentation point (0 h, 6 h and 18 h), set as value 1. Values higher than 1 indicate a higher gene expression than the control, whereas those lower than 1 denote a lower gene expression than the control. *Statistically significant differences (P-value ≤ 0.05) versus their control strains.

Figure 2. Determination of the time required by the mutant and overexpressing strains to ferment 5% (T5), 50% (T50) and 100% (T100) of the initial sugar content in a synthetic must at 12ºC (gray bars) and 28ºC (black bars). Positive and negative values represent the increases and decreases in time (h) of the mutant and overexpressing strains if compared to the control strains (normalized as value 0). The fermentation times of the control strains were: hoQA23 at 12ºC T5 = 62.75 h ± 2.29 h, T50 = 176.75 h ± 3.77 h, T100 = 371.75 h ± 8.66 h; at 28ºC T5 = 17.94 h ± 2.26 h, T50 = 50.12 h ± 3.99 h, T100 = 133.84 h ± 8.66 h and hoQA23-pGREG at 12ºC T5 = 81.75 h ± 5.20 h, T50 = 176.75 h ± 16.75 h, T100 = 361.75 h ± 1.73 h; at 28ºC T5 = 21.85 h ± 1.13 h, T50 = 53.82 h ± 2.10 h, T100 = 136.34 h ± 13.56 h. pGREG (Δtrp1) at 12ºC T5 = 47.75 h ± 1.73 h, T50 = 151.75 h ± 7.40 h, T100 = 341.75 h ± 4.82 h; at 28ºC T5 = 20.12 h ± 1.36 h, T50 = 53.38 h ± 1.36 h, T100 = 148.41 h ± 1.85 h. *Statistically significant differences (P-value ≤ 0.05) as compared with their control strains.

Figure 3. Biplot of the first two PCA components according to the residual amino acids present in synthetic must at a specific density of 1.020 in the 28ºC and 12ºC fermentations. Variables are represented in gray and samples are denoted in black.
Table 1. List of the DNA sequences of the oligonucleotides used in this study.

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</tr>
<tr>
<td>ACT1R</td>
<td>GGCGCAAAATCGATTTCAAA</td>
<td>Real-time quantitative PCR ACT1</td>
</tr>
</tbody>
</table>

a The sequence with homology to the plasmid pAG25 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.

b The sequence with homology to the plasmid pUG6 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.

c The recombination sequences homologous to the plasmid pGREG 505 ends are underlined.

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Table 2. Generation time (GT) of the strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>12ºC</th>
<th>28ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>hoQA23</td>
<td>22.73 ± 0.48</td>
<td>4.82 ± 0.15</td>
</tr>
<tr>
<td>Δtat2</td>
<td>24.20 ± 1.35</td>
<td>4.76 ± 0.10</td>
</tr>
<tr>
<td>Δtrp1</td>
<td>25.56 ± 1.26 *</td>
<td>6.00 ± 0.12 *</td>
</tr>
<tr>
<td>hoQA23 pGREG</td>
<td>25.55 ± 1.16</td>
<td>4.78 ± 0.14</td>
</tr>
<tr>
<td>pGREG TAT2</td>
<td>24.47 ± 0.72</td>
<td>4.99 ± 0.11</td>
</tr>
<tr>
<td>pGREG (Δtrp1)</td>
<td>26.00 ± 2.62</td>
<td>4.71 ± 0.13</td>
</tr>
<tr>
<td>pGREG TAT2 (Δtrp1)</td>
<td>24.11 ± 0.18</td>
<td>4.81 ± 0.09</td>
</tr>
</tbody>
</table>

* Statistically significant differences (P-value ≤ 0.05) as compared with their control strain at the same temperature.

Table 3. Viable cells (CFU/mL (10^7)) at the end of fermentation at 12ºC and 28ºC.

<table>
<thead>
<tr>
<th>Strain</th>
<th>12ºC</th>
<th>28ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>hoQA23</td>
<td>4.40 ± 1.04</td>
<td>7.53 ± 1.72</td>
</tr>
<tr>
<td>Δtat2</td>
<td>5.20 ± 1.76</td>
<td>5.37 ± 0.42</td>
</tr>
<tr>
<td>Δtrp1</td>
<td>2.80 ± 0.14</td>
<td>13.83 ± 1.20 *</td>
</tr>
<tr>
<td>hoQA23 pGREG</td>
<td>7.97 ± 2.76</td>
<td>5.20 ± 2.43</td>
</tr>
<tr>
<td>pGREG TAT2</td>
<td>5.70 ± 2.91</td>
<td>6.57 ± 2.37</td>
</tr>
<tr>
<td>pGREG (Δtrp1)</td>
<td>4.60 ± 2.34</td>
<td>4.43 ± 1.82</td>
</tr>
<tr>
<td>pGREG TAT2 (Δtrp1)</td>
<td>4.37 ± 1.80</td>
<td>4.50 ± 0.44</td>
</tr>
</tbody>
</table>
Table 4. Residual Yeast Assimilable Nitrogen (YAN) in the synthetic must (SM) at 1.020 density of fermentation at 28°C and 12°C, expressed as amino acid (YANaa) and ammonia (YAN NH₄) (mg N /L)

<table>
<thead>
<tr>
<th>Strain</th>
<th>hoQA23</th>
<th>Δtat2</th>
<th>Δtrp1</th>
<th>hoQA23 pGREG</th>
<th>pGREG TAT2</th>
<th>pGREG (Δtrp1)</th>
<th>pGREG TAT2 (Δtrp1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YANaa</td>
<td>85.67 ± 11.34</td>
<td>90.12 ± 2.16</td>
<td>68.18 ± 9.67</td>
<td>102.32 ± 4.50</td>
<td>102.85 ± 13.20</td>
<td>87.57 ± 0.73</td>
<td>78.75 ± 3.90ₐ</td>
</tr>
<tr>
<td>YAN NH₄</td>
<td>2.01 ± 0.43</td>
<td>3.58 ± 2.72</td>
<td>1.56 ± 0.18</td>
<td>1.73 ± 0.07</td>
<td>10.03 ± 7.61</td>
<td>1.62 ± 0.01</td>
<td>1.52 ± 0.03ₐ</td>
</tr>
<tr>
<td>Total YAN</td>
<td>90.80 ± 16.77</td>
<td>93.71 ± 4.88</td>
<td>69.73 ± 9.80</td>
<td>104.11 ± 3.94</td>
<td>112.87 ± 19.43</td>
<td>89.20 ± 0.50</td>
<td>80.27 ± 3.93</td>
</tr>
<tr>
<td>12°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YANaa</td>
<td>102.24 ± 7.92</td>
<td>76.27 ± 15.84</td>
<td>114.33± 8.19ₗ</td>
<td>109.64 ± 0.88</td>
<td>118.96 ± 6.90</td>
<td>120.47 ± 11.10ₗ</td>
<td>105.62 ± 18.93</td>
</tr>
<tr>
<td>YAN NH₄</td>
<td>38.24 ± 5.73ₗ</td>
<td>11.29 ± 9.83</td>
<td>20.62 ± 3.05ₗ</td>
<td>22.88 ± 2.98ₗ</td>
<td>17.72 ± 4.77</td>
<td>14.28 ± 10.19ₗ</td>
<td>14.41 ± 13.79</td>
</tr>
<tr>
<td>Total YAN</td>
<td>140.48 ± 13.65ₗ</td>
<td>87.55 ± 25.67</td>
<td>134.96 ± 11.25ₗ</td>
<td>132.52 ± 2.09ₗ</td>
<td>136.68 ± 5.33</td>
<td>134.75 ± 21.28</td>
<td>120.04 ± 32.00</td>
</tr>
</tbody>
</table>

ₐ Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.

ₗ Significant differences (P-value ≤ 0.05) due to temperature.
Figure 1

![Gene expression bar chart showing relative gene expression over time for pGREG TAT2 and pGREG TAT2 (∆trp1) constructs.

- **pGREG TAT2**
  - Expression levels at 0h, 6h, and 18h.

- **pGREG TAT2 (∆trp1)**
  - Expression levels at 0h, 6h, and 18h.

Significance levels indicated by asterisks (*) for specific time points.
Figure 2

T5

$\Delta tat2$

$\Delta trp1$

pGREG TAT2

pGREG TAT2 ($\Delta trp1$)

Time (hours)

-10 -5 0 5 10 15 20

T50

$\Delta tat2$

$\Delta trp1$

pGREG TAT2

pGREG TAT2 ($\Delta trp1$)

Time (hours)

-20 0 10 20
For Peer Review

T100

Δtat2
Δtrp1
pGREG TAT2
pGREG TAT2 (Δtrp1)

Time (hours)

-80 -60 -20 0 20 40
Figure 3
Table S1: Residual amino acids present in synthetic must at a specific density of 1.020 in the fermentations at 28°C and 12°C, expressed as the mean ± SEM (standard error of the mean) of the amino acid concentration (mg N/L).

<table>
<thead>
<tr>
<th></th>
<th>Aspartic</th>
<th>Glutamic</th>
<th>Asparagine</th>
<th>Serine</th>
<th>Glutamine</th>
<th>Histidine</th>
<th>Glycine</th>
<th>Threonine</th>
<th>Arginine</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hoQA23</td>
<td>0.57 ± 0.09</td>
<td>1.37 ± 0.48</td>
<td>0.1 ± 0.18</td>
<td>0.84 ± 1.00</td>
<td>4.27 ± 3.81</td>
<td>3 ± 0.51</td>
<td>3.53 ± 0.18</td>
<td>1.56 ± 0.44</td>
<td>24.92 ± 2.39</td>
<td>10.57 ± 2.04</td>
</tr>
<tr>
<td>Δtat2</td>
<td>0.68 ± 0.07</td>
<td>1.82 ± 0.28</td>
<td>-</td>
<td>0.27 ± 0.01</td>
<td>6.11 ± 0.46</td>
<td>3 ± 0.15b</td>
<td>3.29 ± 0.16</td>
<td>1.8 ± 0.11</td>
<td>25.43 ± 0.33</td>
<td>12.78 ± 0.67</td>
</tr>
<tr>
<td>Δtrp1</td>
<td>0.31 ± 0.15</td>
<td>0.95 ± 0.35</td>
<td>-</td>
<td>0.21 ± 0.04</td>
<td>1.94 ± 1.10</td>
<td>2.04 ± 0.50b</td>
<td>2.8 ± 0.17a</td>
<td>0.7 ± 1.32</td>
<td>23.28 ± 2.07</td>
<td>6.12 ± 2.00</td>
</tr>
<tr>
<td>hoQA23 pGREG</td>
<td>-</td>
<td></td>
<td>1.63 ± 0.11</td>
<td>-</td>
<td>2.7 ± 0.20</td>
<td>28.74 ± 3.26</td>
<td>-</td>
<td>3.33 ± 0.53</td>
<td>1.83 ± 0.17</td>
<td>24.21 ± 3.13</td>
</tr>
<tr>
<td>pGREG TAT2</td>
<td>-</td>
<td>2.08 ± 0.33</td>
<td>-</td>
<td>3 ± 0.52</td>
<td>28.57 ± 3.51</td>
<td>-</td>
<td>3.59 ± 0.46</td>
<td>2.03 ± 0.38</td>
<td>26.13 ± 4.35</td>
<td>11.55 ± 1.46</td>
</tr>
<tr>
<td>pGREG (Δtrp1)</td>
<td>-</td>
<td>1.62 ± 0.06</td>
<td>-</td>
<td>2.23 ± 0.50</td>
<td>25.44 ± 0.53</td>
<td>-</td>
<td>3.85 ± 0.98</td>
<td>1.5 ± 0.31</td>
<td>21.98 ± 1.20</td>
<td>9.26 ± 1.19</td>
</tr>
<tr>
<td>pGREG TAT2 (Δtrp1)</td>
<td>-</td>
<td>1.07 ± 0.12b</td>
<td>-</td>
<td>1.46 ± 0.27</td>
<td>22.51 ± 2.14</td>
<td>-</td>
<td>3.29 ± 0.07</td>
<td>1.04 ± 0.17</td>
<td>21.52 ± 0.38</td>
<td>7.11 ± 0.61</td>
</tr>
<tr>
<td>12 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hoQA23</td>
<td>1.31 ± 0.18b</td>
<td>2.85 ± 0.24b</td>
<td>0.87 ± 0.01b</td>
<td>2.23 ± 0.48</td>
<td>27.31 ± 4.07b</td>
<td>-</td>
<td>3.14 ± 0.01</td>
<td>1.54 ± 0.38</td>
<td>24.39 ± 0.89</td>
<td>14.43 ± 0.92</td>
</tr>
<tr>
<td>Δtat2</td>
<td>0.56 ± 0.34</td>
<td>1.33 ± 0.78</td>
<td>0.77 ± 0.29</td>
<td>0.75 ± 0.63</td>
<td>15.79 ± 7.71</td>
<td>-</td>
<td>3.33 ± 0.01a</td>
<td>0.53 ± 0.41</td>
<td>22.68 ± 0.43b</td>
<td>8.68 ± 4.61</td>
</tr>
<tr>
<td>Δtrp1</td>
<td>1.49 ± 0.11b</td>
<td>2.88 ± 0.10b</td>
<td>1 ± 0.08b</td>
<td>2.71 ± 0.40b</td>
<td>32.98 ± 3.41b</td>
<td>-</td>
<td>3.4 ± 0.18b</td>
<td>1.77 ± 0.25b</td>
<td>26.79 ± 1.47</td>
<td>15.37 ± 1.05b</td>
</tr>
<tr>
<td>hoQA23 pGREG</td>
<td>1.28 ± 0.04b</td>
<td>2.9 ± 0.02b</td>
<td>0.82 ± 0.20b</td>
<td>1.98 ± 0.05b</td>
<td>31.5 ± 1.56</td>
<td>-</td>
<td>3.3 ± 0.26</td>
<td>1.49 ± 0.16</td>
<td>25.89 ± 1.62</td>
<td>15.15 ± 0.71b</td>
</tr>
<tr>
<td>pGREG TAT2</td>
<td>1.06 ± 0.15b</td>
<td>2.37 ± 0.20b</td>
<td>0.83 ± 0.08</td>
<td>1.57 ± 0.31b</td>
<td>27.6 ± 3.18</td>
<td>-</td>
<td>2.65 ± 1.17</td>
<td>1.22 ± 0.23b</td>
<td>23.75 ± 2.15</td>
<td>14.26 ± 0.59b</td>
</tr>
<tr>
<td>pGREG (Δtrp1)</td>
<td>1.3 ± 0.38b</td>
<td>2.4 ± 0.58</td>
<td>0.63 ± 0.56</td>
<td>2.39 ± 0.96</td>
<td>30.6 ± 6.35</td>
<td>-</td>
<td>3.84 ± 0.30</td>
<td>1.58 ± 0.63</td>
<td>29 ± 1.73b</td>
<td>14.93 ± 2.22b</td>
</tr>
<tr>
<td>pGREG TAT2 (Δtrp1)</td>
<td>0.88 ± 0.78</td>
<td>2.25 ± 0.47b</td>
<td>0.89 ± 0.13b</td>
<td>1.76 ± 0.95</td>
<td>28.18 ± 6.04</td>
<td>-</td>
<td>3.97 ± 0.42</td>
<td>1.17 ± 0.55</td>
<td>26.90 ± 3.20</td>
<td>12.73 ± 3.25</td>
</tr>
</tbody>
</table>

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
b Significant differences (P-value ≤ 0.05) due to temperature.
<table>
<thead>
<tr>
<th></th>
<th>Tyrosine</th>
<th>Valine</th>
<th>Methionine</th>
<th>Cysteine</th>
<th>Isoleucine</th>
<th>Tryptophan</th>
<th>Leucine</th>
<th>Phenylalanine</th>
<th>Ornithine</th>
<th>Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C</td>
<td>0.03 ± 0.01</td>
<td>-</td>
<td>0.67 ± 0.09</td>
<td>1.06 ± 0.1</td>
<td>1.19 ± 0.10</td>
<td>-</td>
<td>0.97 ± 0.15</td>
<td>-</td>
<td>0.18 ± 0.07</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>tAT2</td>
<td>0.04 ± 0</td>
<td>-</td>
<td>0.75 ± 0.09</td>
<td>1.17 ± 0.02</td>
<td>-</td>
<td>0.25 ± 0.02</td>
<td>1.24 ± 0.11</td>
<td>-</td>
<td>0.23 ± 0.12</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>tTP1</td>
<td>0.03 ± 0.1</td>
<td>-</td>
<td>0.68 ± 0.11</td>
<td>1.07 ± 0.05</td>
<td>-</td>
<td>0.29 ± 0.01</td>
<td>1.18 ± 0.13</td>
<td>-</td>
<td>0.26 ± 0.06</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>12°C</td>
<td>0.04 ± 0</td>
<td>-</td>
<td>0.66 ± 0.03</td>
<td>1.07 ± 0</td>
<td>-</td>
<td>0.1 ± 0.0b</td>
<td>1.73 ± 0.06</td>
<td>-</td>
<td>0.17 ± 0.06</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>tAT2</td>
<td>0.03 ± 0.01</td>
<td>-</td>
<td>0.52 ± 0.08</td>
<td>0.98 ± 0.16</td>
<td>-</td>
<td>0.15 ± 0.0b</td>
<td>1.14 ± 0.41</td>
<td>-</td>
<td>0.12 ± 0.01</td>
<td>0.05 ± 0.07</td>
</tr>
<tr>
<td>tTP1</td>
<td>0.05 ± 0</td>
<td>-</td>
<td>0.75 ± 0.08</td>
<td>1 ± 0.06</td>
<td>0.27 ± 0.38</td>
<td>0.49 ± 0.54</td>
<td>0.81 ± 1.14</td>
<td>-</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.11</td>
</tr>
<tr>
<td>12°C</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>hoQA23 pGREG</td>
<td>0.05 ± 0.01</td>
<td>0.83 ± 0.03</td>
<td>0.5 ± 0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.14 ± 0.20</td>
<td>-</td>
<td>2.28 ± 0.19</td>
</tr>
<tr>
<td>pGREG TAT2</td>
<td>-</td>
<td>0.82 ± 0.19</td>
<td>0.71 ± 0.11</td>
<td>2.4 ± 1.29</td>
<td>0.19 ± 0.17</td>
<td>1.11 ± 1.93</td>
<td>11.17 ± 17.20</td>
<td>7.51 ± 11.36</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pGREG TAT2 (∆trp1)</td>
<td>0.03 ± 0.03</td>
<td>0.79 ± 0.23</td>
<td>0.38 ± 0.33</td>
<td>3.36 ± 0.45</td>
<td>0.16 ± 0.27</td>
<td>-</td>
<td>1.08 ± 1.43</td>
<td>1.44 ± 0.03</td>
<td>2.46 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>pGREG TAT2 (∆trp1)</td>
<td>0.02 ± 0.02</td>
<td>0.31 ± 0.53</td>
<td>0.69 ± 0.13</td>
<td>0.61 ± 0.53</td>
<td>1.28 ± 1.28</td>
<td>0.16 ± 0.28</td>
<td>-</td>
<td>0.12 ± 0.21</td>
<td>0.12 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
b Significant differences (P-value ≤ 0.05) due to temperature.
(-) not detected