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Impact of the timing and the nature of nitrogen additions on the production kinetics of fermentative aromas by *Saccharomyces cerevisiae* during winemaking fermentation in synthetic media

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Abstract

During alcoholic fermentation, many parameters, including the nitrogen composition of the must, can affect aroma production. The aim of this study was to examine the impact of several types of nitrogen sources added at different times during fermentation. Nitrogen was added as ammonium or a mixture of amino acids at the beginning of fermentation or at the start of the stationary phase. These conditions were tested with two *Saccharomyces cerevisiae* strains that have different nitrogen requirements. The additions systematically reduced the fermentation duration. The aroma production was impacted by both the timing of the addition and the composition of the nitrogen source. Propanol appeared to be a metabolic marker of the presence of assimilable nitrogen in the must. The production of ethyl esters was slightly higher after the addition of any type of nitrogen; the production of higher alcohols other than propanol was unchanged, and acetate esters were overproduced due to the overexpression of the genes *ATF1* and *ATF2*.Finally the parameter affecting the most the synthesis of beneficial aromas was the addition timing: The supply of organic nitrogen at the beginning of the stationary phase was more favorable for the synthesis of beneficial aromas.

Keywords

Wine fermentation; aroma compounds; nitrogen; alcohol acetyltransferase; online monitoring; *Saccharomyces cerevisiae*

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1. Introduction

Currently, the market offers a wide variety of wines. The main characteristic to discriminate these wines is their sensorial profile constituted by wine aromas (Swiegers et al., 2005; Ugliano et al., 2010). One of the main challenges for wine producers is to control the wine aromatic profile to meet consumer expectations. Among the different volatile molecules involved in wine organoleptic properties, fermentative aromas – generated by the yeast secondary metabolism during alcoholic fermentation – have been identified to be involved in the fruity flavor (Ugliano et al., 2010). Their production depends on many parameters such as the yeast strain (Lambrechts and Pretorius, 2000; Manginot et al., 1998), the must composition in lipids (Rollero et al., 2016; Saerens et al., 2008), the assimilable nitrogen (Barbosa et al., 2012; Hernandez-Orte et al., 2006; Vilanova et al., 2007), or the fermentation temperature (Molina et al., 2007; Mouret et al., 2014b; Rollero et al., 2015).

The nitrogen composition of the must is highly important for aroma synthesis (Swiegers et al., 2005). It has been shown that adding nitrogen to must with a low nitrogen content increases the production of higher alcohols (Barbosa et al., 2009). However, when the assimilable nitrogen concentration exceeds 200 to 300 mgN/L, the production of higher alcohols decreases (Mouret et al., 2014b; Vilanova et al., 2007). Propanol does not have the same behavior as the other higher alcohols. Its production is proportional to the initial amount of nitrogen (Mouret et al., 2014b). Finally, the concentration of acetate esters and ethyl esters increases with the initial quantity of assimilable nitrogen (Hernandez-Orte et al., 2006; Torrea et al., 2011).

The effects observed after nitrogen addition in the stationary phase differ depending on the studies, probably because of the use of different fermentation conditions. For example, Hernandez-Orte et al. (2006) showed that addition at mid-fermentation had no significant impact on the production of higher alcohols but led to greater increases in the production of

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esters than addition at the beginning of the process. In contrast, Jiménez-Martí et al. (2007) observed a decrease in higher alcohol production after addition during the stationary phase, but there was no effect on the production of acetate esters.

The effect of the type of nitrogen on aroma production also depends on the yeast strain and fermentation conditions. Webster et al. (1993) demonstrated that addition of amino acids led to an increased production of higher alcohols compared to addition of ammonium, but Barbosa et al. (2012) found that isoamyl alcohol production is higher when ammonium is added. Contradictory observations were also observed for acetate and ethyl esters (Barbosa et al., 2012; Torrea et al., 2011), indicating that no simple relationship can be established between the nature of the nitrogen supply and the production of esters. It is thus difficult to draw conclusions on the effect of nitrogen addition on aroma production, and much remains unknown about the impact of nitrogen on yeast metabolism.

The aim of this study was to fully characterize the impact of nitrogen addition on the formation of volatile compounds during alcoholic fermentation by utilizing innovative strategies such as the online monitoring of aroma production. We studied two types of nitrogen: ammonium and amino acids. The additions were made either at the beginning of fermentation or at the start of the stationary phase. We also investigated the impact of these additions on the metabolic pathways of the aroma production and their regulation. Understanding the impact of nitrogen on the metabolism of *S. cerevisiae* in alcoholic fermentation allows better management of this nutrient to produce volatile compounds.

2. Materials & Methods

2.1. Yeast strains

The *S. cerevisiae* yeast strains used in this study are the commercial strains Lalvin EC1118® and Lalvin CY3079® (Lallemand SA, Montreal, Canada). These two strains have different

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nitrogen requirements, with Lalvin EC1118® requiring less nitrogen than Lalvin CY3079®. Fermentation tanks were inoculated with 10 g/hL active dry yeast previously rehydrated for 30 minutes at 37°C in a 50 g/L glucose solution (1 g of dry yeast diluted in 10 mL of this solution).

2.2. Fermentation media

Fermentation was carried out in synthetic medium (SM) that simulates standard grape juice (Bely et al., 1990). The SM used in this study contained 200 g/L of sugar (100 g/L of glucose and 100 g/L of fructose). The concentrations of weak acids, salts and vitamins were identical to those described by Rollero et al. (2016). The pH of the medium was adjusted to 3.3 with 10 M NaOH.

The must contained 100 mg/L of assimilable nitrogen. The nitrogen source was composed of ammonium chloride and amino acids. The composition of the stock solution of amino acids was the same to that described by Rollero et al. (2016).

The SM medium was initially supplemented with 2 mg/L of phytosterols (85451, Sigma Aldrich) to satisfy the lipid requirements of yeast cells during anaerobic growth. The stock solution was the same to the one described by Rollero et al. (2016).

Different types of nitrogen were added to this synthetic must: di-ammonium phosphate (DAP) or a mixture of amino acids to a concentration of 70 mg N/L. These additions were performed initially or during the stationary phase (at 85 g/L of consumed sugar).

2.3. Fermentation conditions

Fermentations were carried out in 10 L stainless steel tanks at 24°C. The amount of CO_2 released was measured accurately and automatically with a gas mass flow meter to calculate

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the rate of CO_2 production (dCO_2/dt). Anaerobiosis was obtained by bubbling argon into the medium.

Each fermentation was performed once. However, we previously determined that experiments conducted with this online monitoring system yield highly reproducible results (Mouret et al., 2012; Mouret et al., 2014b).

For the quantitative PCR, fermentations were achieved in 1 L fermenters at 24°C in triplicate. As described by Rollero (2015), fermenters of 1 L and 10 L were designed to allow scale-up without any effect on the outcome of fermentation. The fermenters were equipped with fermentation locks to maintain anaerobiosis with continuous magnetic stirring (500 rpm). The CO_2 release was followed by automatic measurement of fermenter weight loss every 20 minutes. The rate of CO_2 production was calculated by polynomial smoothing of the last ten values of CO_2 production. The frequent acquisition of CO_2 release values and highly precise bioreactor weighing enabled accurate CO_2 production rates to be calculated with good reproducibility and a small variation coefficient: $(dCO_2/dt)_{max} = 0.8\%$ (Bely et al., 1990).

2.4. Cell population

During fermentation, the cell population was determined by using a Coulter counter (Model Z2, Beckman-Coulter, Margency, France) fitted with a 100 µm aperture probe.

2.5. Measurement of assimilable nitrogen

The ammonium concentration was determined enzymatically (R-Biopharm, Darmstadt, Germany).

The free amino acid content of the must was determined by cation exchange chromatography with post-column ninhydrin derivatization (Biochrom 30, Biochrom, Cambridge, UK) as described by Crepin et al. (2012).

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2.6. Analysis of volatile compounds

The concentrations of volatile compounds in the headspace of the tank were measured with an online GC device. Headspace gas was pumped from the tank at a flow rate of 14 mL/min through a heated transfer line. Carbon compounds were concentrated in a cold trap (Tenax TM) for 6 minutes, desorbed at 160°C for 1 minute, and analyzed with a Perichrom PR2100 GC coupled to a flame ionization detector (Alpha MOS, Toulouse, France). The GC method and the calibration procedure were performed as described previously by Mouret et al. (2014a and b).

The results are expressed as total production, representing the sum of the concentration measured in the gas phase and the concentration in the liquid phase as described by Mouret et al. (2014a and b). The total production value represents the capability of the yeast to produce a volatile compound, independently of whether the subsequent fate of this compound was accumulation in the liquid phase or evaporation.

2.7. Data processing and statistical analyses

Statistical analysis was performed with R software, version 3.1.1.

We obtained two datasets in which each variable of interest is a curve along time (h) that we expressed in terms of consumed sugar (g/L). We chose to summarize these two datasets by modeling each curve with an adequate model and then extracting criteria of interest.

First, for each condition, the biomass was modeled with a Weibull model using the drc package (Ritz and Streibig, 2005). The four-parameter Weibull model is written as follows:

$$f(x) = c + (d - c)\left[1 - exp^{-exp^{[b\,(\ln(x) - \ln(e))]}}\right] \qquad (Equation\ 1)$$

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This four-parameter ascending function is asymmetric with an inflection point at time e. For each modeled function, we extracted several criteria of interest: µmax, defined as the maximum of the ratio f'(t)/f(t) for each t expressed in h^{-1}, the inflection point, expressed in terms of consumed sugar g/L, and the maximum biomass, expressed in 10^6 cells.

For this parametric model, the normality of residual distributions and homogeneity of variance were studied with standard diagnostic graphs. No violation of the assumptions was detected.

Each volatile compound under each condition was then modeled with a non-parametric model using the cellGrowth package (Gagneur and Neudecker, 2012). The model used is a local regression and allows for the extraction of the inflection point expressed in consumed sugar (g/L), the maximal production in mg/L and the maximal rate (maximum of the first derivative in mg/L/h). To calculate the specific rate, we divided the first derivative of the model (the rate) by the population as estimated above.

To provide an overview of the dataset, principal component analysis (PCA) was carried out with the FactoMineR package (Le et al., 2008).

2.8. RNA extraction

For each fermentation condition, 1 x 10^9 cells were harvested by centrifugation at 1,000*g* for 5 min at 4 °C. The cell pellets were washed with DEPC-treated water and then frozen in methanol at -80 °C. Total RNA was extracted with the TRIzol reagent (Gibco BRL, Life Technologies) and purified with an RNeasy kit (Qiagen).

2.9. Retro-transcription

First-strand cDNA was synthesized using 0.5 µg of total RNA by priming with an oligo(dT) anchor at 42 °C for 50 min using Superscript-II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

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2.10. Primers and quantitative PCR

All of the primers were designed using Primer 3 software. The amplification efficiency was calculated using the dilution method (Rasmussen, 2001). A description of the genes, primers, and efficiencies are shown in Table 1.

Real-time PCR was performed in 96-well plates on an Applied Biosystems 7300 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR Green as the fluorophore. Reactions were carried out in a 20 µL volume that contained 5 µL of cDNA, 3 µM forward and reverse primers, and 12.5 µL of 2x Power SYBR Green PCR Master Mix. Each sample was analyzed in triplicate, and a no-template control for each primer was included in all real-time plates. Amplifications were performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 10 s, and 60 °C for 1 min. At the end of the amplification cycle, a melting analysis was carried out to verify the absence of non-specific amplification. The expression level of a given gene was reported as the quantification cycle (Cq), corresponding to the number of cycles required to reach the pre-determined threshold fluorescence. The threshold values were obtained using the automated setting of the instrument software (baseline subtracted curve fit data). The data, expressed as Cq, were imported into a Microsoft Excel data sheet for subsequent analysis.

2.11. Analysis of target genes

The expression of two target genes, *ATF1* and *ATF2,* was analyzed (Table 1). The genes were chosen for their involvement in the conversion of higher alcohols into acetate esters. The relative quantification of the mRNA was performed by normalizing the expression value transformed in relative copy numbers (obtained from Cq values and considering the efficiency) to the geometric mean of the value of a reporter gene. The reporter gene was SCR1, a structural component of the signal recognition particle (Hann and Walter, 1991). In

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the calculation of the relative copy number from raw Cq, amplification efficiencies were considered.

3. Results and discussion

We investigated the impact of the addition of 70 mg/L of YAN on the fermentation kinetics and on the main volatile compound production. We tested two different sources of nitrogen: a mineral source as di-ammonium phosphate (DAP) and an organic one in the form of an amino acid mixture. Additions were performed either at the beginning of fermentation or at the start of the stationary phase (approximately 85 g/L of sugar consumed). The fermentative kinetic and the production rate of eight aroma compounds were monitored online. These compounds are propanol, isoamyl alcohol, isobutanol, isoamyl acetate, isobutyl acetate, ethyl acetate, ethyl hexanoate, and ethyl octanoate.

The nitrogen and sugars were exhausted at the end of all of the fermentations. Final concentrations of ethanol, glycerol and acetate were identical for all the conditions (data not shown).

3.1. Effect of nitrogen addition on the fermentation kinetics

We compared the impact of different additions of nitrogen on the fermentation kinetics (Figure 1).

The length of the lag phase was not impacted by the nitrogen content of the medium. In addition, the growth phases of the fermentations in which nitrogen was added in the stationary phase were the same as those of the control fermentation. This confirms that the fermentations are very reproducible.

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As expected (Bely et al., 1990; Mendes-Ferreira et al., 2004), nitrogen addition at the beginning of fermentation led to an increase of the maximal CO_2 production rate. Regardless of the nature of the added source, this value was equal to 0.79 g/L/h for the control fermentation and increased to 1.4 g/L/h with an initial addition of nitrogen. We compared the impact of different nitrogen additions on the fermentation kinetics (Figure 1).

The production of biomass was also 1.3-fold higher when more nitrogen was provided in the must (Bell and Henschke, 2005; Bely et al., 1990).

The consequences of nitrogen addition during the stationary phase were very different from those observed following an initial addition to the must. Adding nitrogen at the start of the stationary phase resulted in an immediate increase of the fermentation activity up to a maximum of 0.9 g/L/h of CO_2 produced, measured when all of the nitrogen added was exhausted. However, the number of cells remained the same after the addition, as previously observed by Bely et al. (1990). Overall, the additions of nitrogen substantially reduced the fermentation duration (Table 2), as reported previously (Bell and Henschke, 2005). It should be noted that the lowest fermentation times were obtained when nitrogen was added during the stationary phase. This last observation confirms the value of adding nitrogen during this phase, especially in the case of musts with low levels of phytosterols. Thus, adding nitrogen after the cell growth phase activates the yeast metabolism without increasing the cell population and consequently without diluting the lipid content of the yeasts. Therefore, the improved fermentation performances obtained with addition of nitrogen at the beginning of the stationary phase compared with an initial addition are likely due to a better resistance of yeasts to ethanol at the end of fermentation (Sablayrolles et al., 1996).

In summary, both the fermentative kinetics (Figure 1) and the biomass production are only modulated by the timing of the addition of nitrogen, regardless of the nature of the source added (Figure 1 and Table 2).

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262

263 3.2. Impact of nitrogen addition on volatile compound production

264 The data presented for aroma compounds are the total production, *i.e.*, the sum of the liquid
265 concentration and losses in the gas, because it is representative of the yeast metabolism
266 (Mouret et al., 2014a).

267 *3.2.1 Ethyl esters*

268 We followed the production rate of two ethyl esters: ethyl hexanoate and ethyl octanoate
269 (Figure 2a, b, c and d).

270 After nitrogen addition at the beginning of the fermentation, the total production of ethyl
271 hexanoate and octanoate increased moderately (+15% on average for the different conditions).
272 These variations are consistent with a mathematic model developed by Rollero et al. (2015)
273 for ammonium additions. This model predicts the production of the principal aroma
274 compounds depending on the temperature and the amount of lipid and nitrogen in the must.
275 With our parameters, this model predicts an increase of 10% for these two compounds from
276 100 to 170 mg/N/L.

277 We showed that the consequences of nitrogen supply in the must were comparable for the two
278 ethyl esters studied. However, when the nitrogen is added during the stationary phase, the
279 behavior of each ester was different. An addition during the stationary phase increased the
280 final concentration of ethyl hexanoate to the same extent as an initial addition. A higher
281 synthesis of ethyl hexanoate following the addition of nitrogen was clearly visible in the
282 evolution of the specific production rate of this ester (Figure 3). After the addition, this rate
283 doubled compared to the control fermentation. In contrast, the addition of nitrogen in the
284 stationary phase had no impact on the production of ethyl octanoate (Figure 2d).

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The low impact of the addition of nitrogen on ethyl ester production is likely due to the absence of causal relationships between the metabolic pathways of ethyl ester synthesis and nitrogen metabolism. However, the nitrogen availability, inducing changes in the redox balance of the cell, could modify the intracellular level of precursors, especially acetyl-coA and further acyl-CoA derivatives (Bloem et al., 2016) or lipid synthesis due to the biomass increase following the addition of nitrogen (Daum et al., 1998). This could explain the variation of ethyl ester production depending on the time of the addition of nitrogen, as adding nitrogen during the stationary phase resulted in a reduced increase in the final population compared to an initial supplementation and consequently resulted in limited changes in acyl-CoA availability.

Furthermore, these modifications could be too small to be sufficient to trigger an increase of ethyl octanoate synthesis, which requires the addition of two additional carbons compared to ethyl hexanoate.

3.2.2. Higher alcohols

3.2.2.1 Propanol

Propanol production is different than that of the other higher alcohols, as it is strongly associated with the concentration of available nitrogen in the must (Mouret et al., 2014b). During the control fermentation or fermentations with nitrogen added initially, propanol production ended when the assimilable nitrogen was exhausted (Figure 4). The production of propanol re-started immediately after nitrogen addition during the stationary phase and stopped again when there was no more nitrogen in the medium.

These observations, confirming that propanol is a marker of nitrogen availability in the must (Mouret et al., 2014b), also provided new insights on the activity of the metabolic pathway involved in the synthesis of this compound. We first observed that the nature of added nitrogen source, whether mineral or organic, has a direct impact on the amount of propanol

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produced. An ammonium addition induced greater production of propanol than an amino acid addition (Figure 2e and f). This phenomenon was especially pronounced during additions in the stationary phase: the final propanol production was increased 4.3-fold after an ammonium addition in the stationary phase, compared to a 2-fold increase after the addition of amino acids (Table 2). These results could be explained by a different management of nitrogen by cells depending on whether the source was organic or mineral. Thus, In particular, the assimilated ammonium is entirely directed toward the common nitrogen pool, where it is combined with α-ketoacids to produce the amino acids required for cell growth (Magasanik and Kaiser, 2002). Amino acids are also largely directed toward the common nitrogen pool, but part of it is directly assimilated without transformation (Crépin et al., 2014). Therefore, for a given quantity of assimilable nitrogen, the nitrogen content incorporated into the common pool is lower when organic nitrogen is added compared to a similar addition of ammonium. This causes a reduction of the synthesis of the ketoacid precursor of propanol and thus results in a lower production of propanol. These data demonstrate that propanol can also be considered as a marker of the quality of available nitrogen (mineral or organic).

Then, we observed that propanol production was higher after nitrogen addition during the stationary phase than at the beginning of the fermentation (Figure 2f). This likely reflects a different management of the nitrogen resources depending on the timing of the addition. Propanol is synthetized from α-ketobutyrate derived from threonine. However, α-ketobutyrate is also a precursor of isoleucine. Propanol synthesis could be a way to degrade unused α-ketobutyrate (Bisson, 1991). During cell growth, the demand for protein synthesis is high, especially since the nitrogen addition at the beginning of the fermentation leads to an increase in biomass production. A large part of α-ketobutyrate is used to produce isoleucine. In contrast, the immediate demand of proteins is lower during the stationary phase when cell

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growth has stopped. Because the isoleucine needs are lower, the α-ketobutyrate flux could be re-oriented to the production of propanol.

3.2.2.2 Other higher alcohols

The impact of nitrogen addition on the other higher alcohols was different from that on propanol.

In contrast to propanol, after a nitrogen addition at the beginning of fermentation, the total productions of isoamyl alcohol and isobutanol were almost the same as those reached during control fermentation (Figure 2g and i). These results are consistent with the predictions of the mathematical model developed by Rollero et al. (2015), which predicted that the total final concentrations obtained for the two higher alcohols studied would be identical with 100 mgN/L and 170 mgN/L of assimilable nitrogen.

In addition, the total productions of isoamyl alcohol and isobutanol were almost identical to those obtained during the control fermentation regardless of the time or type of nitrogen addition (Table 2, Figure 2g, h, i and j).

This absence of a difference could be explained by the fact that α-ketoacids that are isobutanol and isoamyl alcohol precursors are produced from both central carbon metabolism (CCM) and the catabolism of amino acids by the Ehrlich pathway (Bell and Henschke, 2005; Hazelwood et al., 2008). Previous studies have demonstrated that the CCM contribution to the synthesis of higher alcohols is much more important than the contribution of the amino acids (Rollero, 2015). We can assume that there is a balance between the amount of α-ketoacids supplied by CCM and the amount of α-ketoacids converted into amino acids for the synthesis of proteins. Therefore, during an addition of nitrogen, the supplementary flux of α-ketoacids related to the addition would be negligible compared to the flux from CCM (Crépin et al.,

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2017; Rollero et al., 2017). The α-ketoacid pool available would then remain the same and would not change the final production of higher alcohols.

3.2.3. Acetate esters

Despite their very limited incidence in the production of branched alcohols, nitrogen supplementation, regardless of the nature of the added compound and the times of addition, resulted in a large overproduction of acetate esters (Figure 2i and j). This impact was especially pronounced when adding nitrogen at the beginning of the stationary phase: the final concentration of isoamyl acetate was 2.69 mg/L after a DAP initial addition but 4.09 mg/L after a DAP addition at the beginning of the stationary phase (Table 2). Furthermore, an addition of amino acids at the beginning of the stationary phase led to an increase of 20% of the production of isoamyl acetate in comparison with an addition of DAP (Figure 2j). However, the most important parameter for the production of acetate esters in our study was the timing of the addition (Table 2).

This systematic increase of the synthesis of acetate esters as a consequence of nitrogen addition while the concentration of the corresponding higher alcohol precursors remained unchanged was surprising. Thus, we focused on the bioconversion between these two types of molecules. A significant increase of the conversion yields of higher alcohols into corresponding acetate esters was noted when nitrogen was added (Figure 5a and b). After the addition of nitrogen at the beginning of the fermentation (either ammonium or amino acids) (Figure 5a), the yields were multiplied by 2 and by 10 after addition during the stationary phase (Figure 5b). These observations suggest that the effect of nitrogen is focused on the enzymes of the group of alcohol acetyltransferases (Atf1p and Atf2p) that catalyze the conversion of higher alcohols into acetate esters (Verstrepen et al., 2003).

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To identify whether the genes coding for these enzymes are up-regulated as a consequence of nitrogen addition, we measured the transcription level of the genes *ATF1* and *ATF2* by real-time PCR experiments to investigate the impact of the addition of nitrogen in the form of DAP or a mixture of amino acids during the stationary phase on their expression.

As a consequence of nitrogen addition, the transcription of *ATF1* and *ATF2* was significantly increased in comparison with the control (Figure 6). The overexpression was greater for *ATF2* than for *ATF1*. For the amino acid addition, the expression ratio compared to the reference gene was equal to 234% for *ATF2* and 184% for *ATF1*.

A difference in the levels and kinetics of expression of these two genes was observed depending on the nature of the nitrogen source added. The addition of DAP caused only a small increase in the expression of the gene *ATF1* (141%), which remained constant during the five hours following this addition (Figure 6a). In contrast, the addition of a mixture of amino acids led to a peak of expression of this gene two hours after the addition. We observed a strong increase in the expression of the gene *ATF2* 30 min after the nitrogen addition for both nitrogen sources (Figure 6b). Nevertheless, this expression level was maintained for the addition of DAP but decreased sharply after five hours following the addition of amino acids. These differences according to the types of nitrogen sources added could explain the differences in the production of esters acetate observed when adding amino acids or DAP (Figure 2i and j).

The transcription of the gene *ATF1* is regulated by different environmental parameters (Fujiwara et al., 1999) (Figure 7). In particular, it was shown that the promoter of the gene *ATF1* contains a binding site for the Rap1p protein. This protein is known to be a target of the Ras/cAMP/PKA pathway, which is the main glucose signaling pathway in yeast (Fujiwara et al., 1999). However, another signaling pathway is known to target the same genes: the FGM (fermentable-growth-medium-induced) pathway, which is active when all of the nutrients

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needed for growth, including nitrogen, are present in the culture medium. The main protein involved in this pathway is the protein kinase Sch9p, which has similarities with the catalytic subunit of the cAMP-dependent protein kinases present in the Ras/cAMP/PKA pathway (Verstrepen et al., 2003). It has been shown that the Sch9p protein plays a role in the expression of *ATF1*, as its deletion leads to a decrease in the expression of the gene (Fujiwara et al., 1999). These authors suggested that Sch9p would act on the activity of Rap1p and therefore on the transcription of *ATF1*. With the results we obtained in RT-PCR, we confirmed the nitrogen effect on the transcription of the gene *ATF1,* and we highlighted that this effect also exists even more strongly for the gene *ATF2*. We can thus suggest that the regulation of transcription of this gene was carried out via the same signaling pathway as for *ATF1*. Moreover, it seems that *ATF2* is more sensitive to sudden changes in nitrogen availability during fermentation than *ATF1*, as its activity increased more significantly. Finally, the implication of nitrogen addition on the availability of acetyl-coA, which is required for the synthesis of acetate esters (Bell and Henschke, 2005), can also be discussed. Indeed, the Michaelis constant Km of Atf1p for acetyl-coA is very low (0.025 mM) (Mason and Dufour, 2000). Therefore, a slight change of acetyl-coA availability could have an important effect on ester synthesis by Atf1p. This could explain why more acetate esters are produced during the stationary phase than at the beginning of fermentation. During the growth phase, acetyl-coA would mainly be used for biomass synthesis. In contrast, after nitrogen addition in the stationary phase, acetyl-coA would be more available for aroma production because there is no more biomass production.

3.3. Impact of nitrogen on different yeast strains

Fermentations were conducted with two *S. cerevisiae* strains having different nitrogen requirements, which correspond to the amount of nitrogen that must be added during

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fermentation to maintain a constant fermentation rate (Julien at al., 2000). The production of the aromas studied by these two strains is represented on a PCA (Figure 8). The two first PCA axes accounted for 80.9% of the total variation for Lalvin EC1118® (Figure 8a) and 80.4% for Lalvin CY3079® (Figure 8b). For the two strains, the repartitions of the fermentation conditions on the PCA were comparable, indicating that the effects of the nitrogen management on the synthesis of fermentative aromas are similar for Lalvin EC1118® and Lalvin CY3079®. However, it should be noted that the effect of the type of nitrogen added at the beginning of fermentation was less visible with Lalvin CY3079®, as the individuals are closer on the PCA. The principal parameter discriminating the fermentation conditions was the timing of the addition, and the compounds most impacted were propanol and acetate esters.

4. Conclusion

The objective of this study was to examine the impact of the nitrogen source (ammonium or amino acids) and the timing of the addition of nitrogen on the aroma production of two *Saccharomyces cerevisiae* strains during winemaking conditions.

The two *S. cerevisiae* strains had different nitrogen requirements and aromatic profiles, but the impacts of nitrogen supply on their aroma metabolism were quite similar. From a general point of view, the impact of the timing of the addition of nitrogen on aroma production was higher than that of the nitrogen composition, even if adding organic nitrogen slightly increased the acetate ester synthesis compared to the supply of ammonium.

We confirmed that propanol is a metabolic marker of the presence of available nitrogen in the must and, more precisely, of the amount of intracellular nitrogen available for the *de novo* synthesis of amino acids. In contrast, the production of other higher alcohols was not affected

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by the additions of nitrogen. Ethyl ester production was slightly increased after the addition of nitrogen, perhaps because of a change in the availability of acetyl-coA. The strongest impact of the nitrogen could be observed for the acetate esters. In particular, their production increased greatly after the addition of nitrogen, especially during the stationary phase. We showed that the overproduction of these specific volatile compounds was due to an increase of the production yields of acetate esters from corresponding higher alcohols resulting from an overexpression of the genes *ATF1* and *ATF2* that encode for this enzymatic bioconversion activity.

In the future, it would be interesting to confirm the effect of nitrogen management on other strains to evaluate the generic aspect of the results obtained. In addition, these experiments should be reproduced on natural must to validate that the differences in aroma concentrations observed in this study have a perceptible impact on the sensory profile of the wine. Different addition quantities could be tested, as the nitrogen concentration has an important impact on the aroma production. It would also be interesting to study the metabolic fluxes impacted by nitrogen addition in detail, especially the production of propanol from threonine, to explain the production differences observed depending on the nitrogen source. However, these findings open up new perspectives for the development of innovative strategies aimed at maximizing the production of positive aromas through adapted nitrogen management.

5. Acknowledgments

We thank Evelyne Aguera, Magaly Angenieux, Christian Picou and Martine Pradal for technical assistance.

6. Conflict of interest

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611 **Tables**

612 Table 1: Target genes used in qPCR.

Genes	Molecular function (SGD curate)	Reference	Primer sequence (5' -> 3')
ATF1	Alcohol acetyltransferase activity	(Fujii et al., 1994)	F: ATGATTCAGAATGGGCATGCT R: CTCCATATGTGCAGAAGTTTCGA
ATF2	Alcohol acetyltransferase activity	(Nagasawa et al., 1998)	F: TTCATTCAAGCGTGCTTTCTTG R: CGTTGCTTGGAATAGCCACAT
SCR1	RNA subunit of the Signal Recognition Particle	(Felici et al., 1989)	F: TCTGGTGCGGCAAGGTAGTT R: CACCTTTGCTGACGCTGGAT

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Table 2: Fermentation parameters and final aroma concentrations measured for fermentations with Lalvin EC1118®.

		Fermentation duration (h)	Maximal population (Ȼ/mL)	First peak of dCO_2/dt (g/L/h)	Second peak of dCO_2/dt (g/L/h)	Final concentration (total production) (mg/L)							
						Propanol	Isoamyl alcohol	Isobutanol	Isoamyl acetate	Isobutyl acetate	Ethyl acetate	Ethyl hexanoate	Ethyl octanoate
Control		310	5.02E+07	0.79	-	5.60	193.76	24.28	1.38	0.10	32.15	1.78	2.73
Addition at T_0	**DAP**	270	6.79E+07	1.36	-	10.65	199.33	27.69	2.69	0.22	36.80	1.94	3.25
	AA	270	6.78E+07	1.40	-	8.26	163.56	25.06	2.93	0.38	38.73	2.07	2.98
Addition in stationary phase	**DAP**	215	5.33E+07	0.75	0.91	24.17	189.31	19.57	4.09	0.24	49.12	2.17	2.62
	AA	220	5.35E+07	0.75	0.90	11.67	195.63	23.13	4.92	0.28	48.40	2.02	2.48

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Figures

Figure 1: Changes in the fermentation kinetics depending on the addition of nitrogen with Lalvin EC1118®: control without addition (green curve); addition at the beginning of fermentation of DAP (dark blue curve) or amino acids (red curve); addition during the stationary phase of DAP (light blue curve) or amino acids (orange curve). The arrow indicates the timing of addition during the stationary phase.

Figure 2: Total production by Lalvin EC1118® of ethyl hexanoate, ethyl octanoate, propanol, isoamyl alcohol and isoamyl acetate during the fermentation with nitrogen additions at the beginning of the fermentation (respectively, a, c, e, g and i) and during the stationary phase (b, d, f, h and j). Control fermentation (green curve); addition of DAP at the beginning of the fermentation (dark blue curve); addition of DAP during the stationary phase (light blue curve); addition of amino acids at the beginning of the fermentation (red curve); addition of amino acids during the stationary phase (orange curve); Arrows represent the additions during the stationary phase.

Figure 3: Changes in the specific rate of ethyl hexanoate production after an addition during the stationary phase. Control fermentation (green curve); addition of DAP (light blue curve); addition of amino acids (orange curve). The arrow indicates the timing of addition during the stationary phase.

Figure 4: Propanol production (orange curve) and assimilable nitrogen consumption (purple crosses) by Lalvin EC1118® during a fermentation with the addition of amino acids in the stationary phase. The arrow represents the addition during the stationary phase.

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Figure 5: Changes in the total production of isoamyl acetate as a function of the total production of isoamyl alcohol after nitrogen addition at the beginning of fermentation (a) or in the stationary phase (b) with Lalvin EC1118®. Control without addition (green curve); addition at the beginning of fermentation of DAP (dark blue curve) or amino acids (red curve); addition during the stationary phase of DAP (light blue curve) or amino acids (orange curve). The arrow indicates the timing of addition during the stationary phase.

Figure 6: *ATF1* (a) and *ATF2* (b) expression of Lalvin EC1118® after nitrogen addition measured by qPCR. Control (green bar); addition of DAP (light blue bar); addition of amino acids (orange bar).

Figure 7: Diagram of the transcriptional regulation of *ATF1* by glucose and nitrogen (from Fujiwara et al., 1999).

Figure 8: Principal component analysis of total aroma production at 180 g/L of consumed sugar for the strains Lalvin EC1118® (a) and Lalvin CY3079® (b). Control fermentation (green circle); initial additions (purple circle); addition during the stationary phase (blue circle). Addition of ammonium (NH_4) or amino acids (AA). EH = ethyl hexanoate, EO = ethyl octanoate, PR = propanol, IA = isoamyl alcohol, IB = isobutanol, IAA = isoamyl acetate, IBA = isobutyl acetate, EA = ethyl acetate.

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Highlights

Nitrogen content of the must impacts aroma production in alcoholic fermentation.

The timing of addition and the source of nitrogen had an effect on aroma production.

Acetate esters were the most impacted by nitrogen addition.

After a nitrogen addition in stationary phase, *ATF1* and *ATF2* were overexpressed.

The best results were obtained when adding organic nitrogen in stationary phase.

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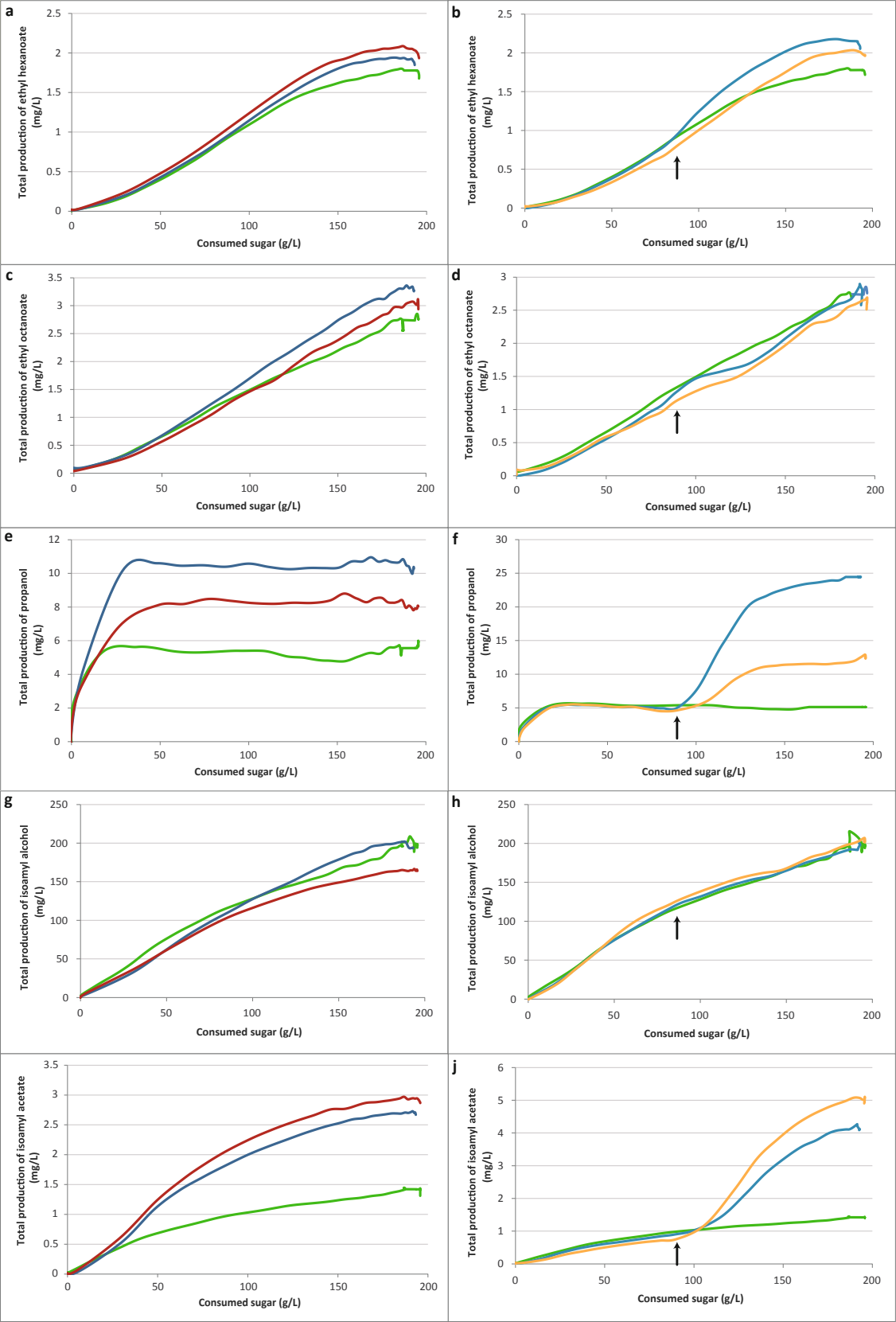
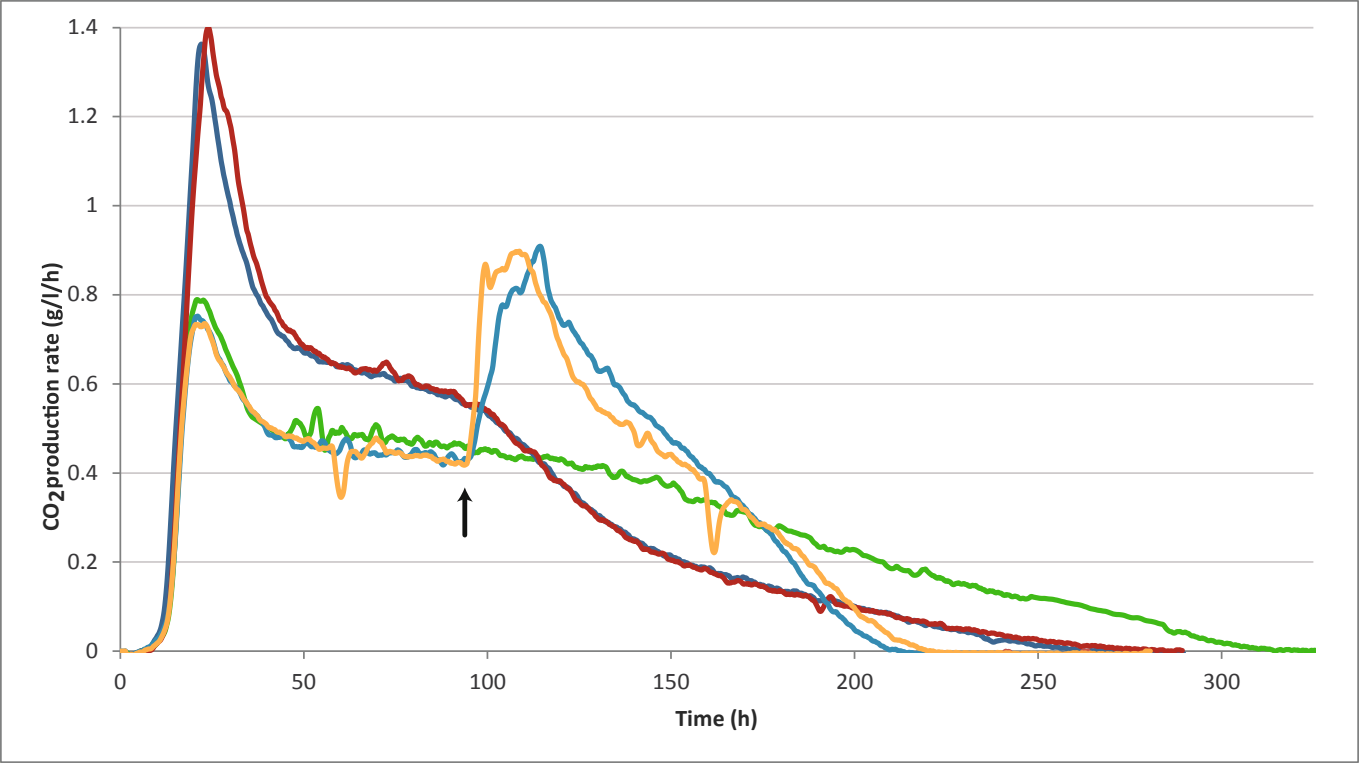
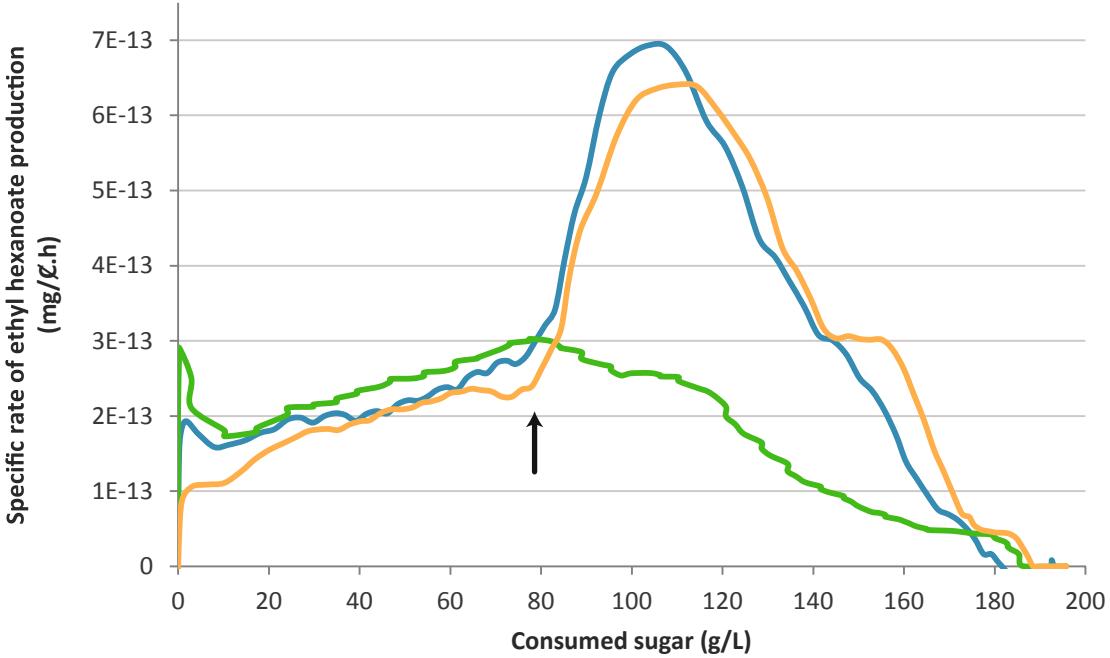
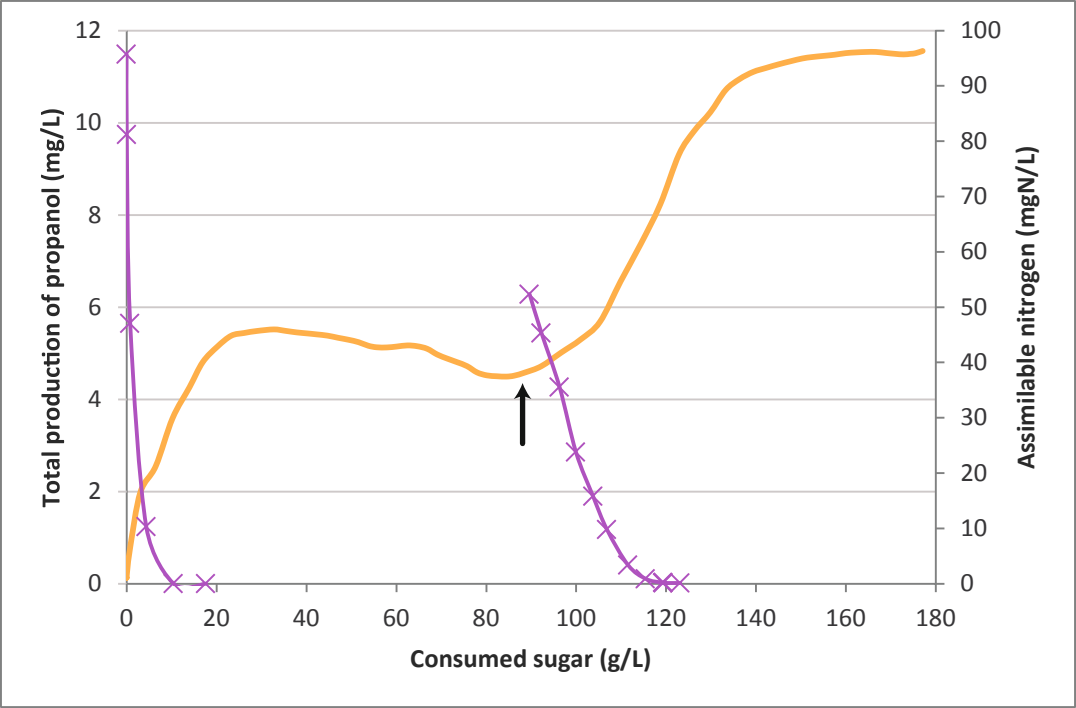
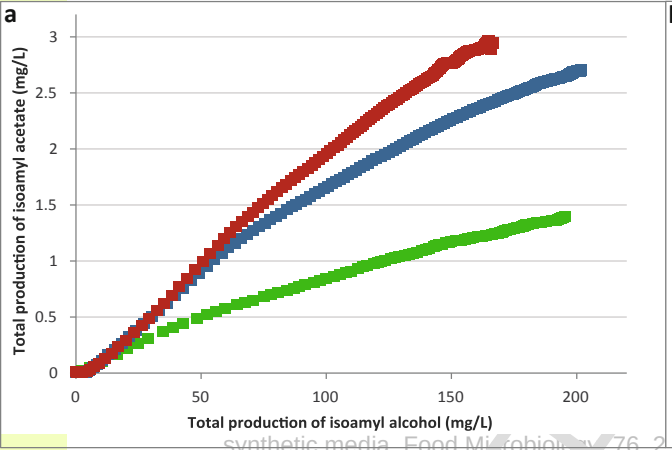
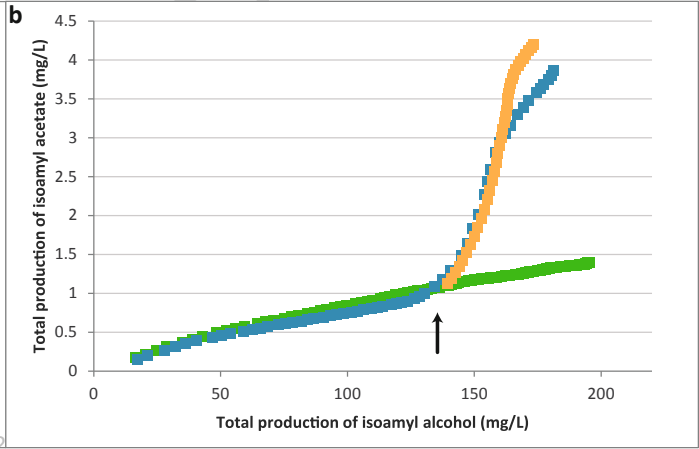

a
Total production of ethyl hexanoate (mg/L)
Consumed sugar (g/L)
b
Total production of ethyl hexanoate (mg/L)
Consumed sugar (g/L)
c
Total production of ethyl octanoate (mg/L)
Consumed sugar (g/L)
d
Total production of ethyl octanoate (mg/L)
Consumed sugar (g/L)
e
Total production of propanol (mg/L)
Consumed sugar (g/L)
f
Total production of propanol (mg/L)
Consumed sugar (g/L)
g
Total production of isoamyl alcohol (mg/L)
Consumed sugar (g/L)
h
Total production of isoamyl alcohol (mg/L)
Consumed sugar (g/L)
Total production of isoamyl acetate (mg/L)
Consumed sugar (g/L)
j
Total production of isoamyl acetate (mg/L)
Consumed sugar (g/L)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
CO_2 production rate (g/l/h)
0
50
100
150
200
250
300
Time (h)

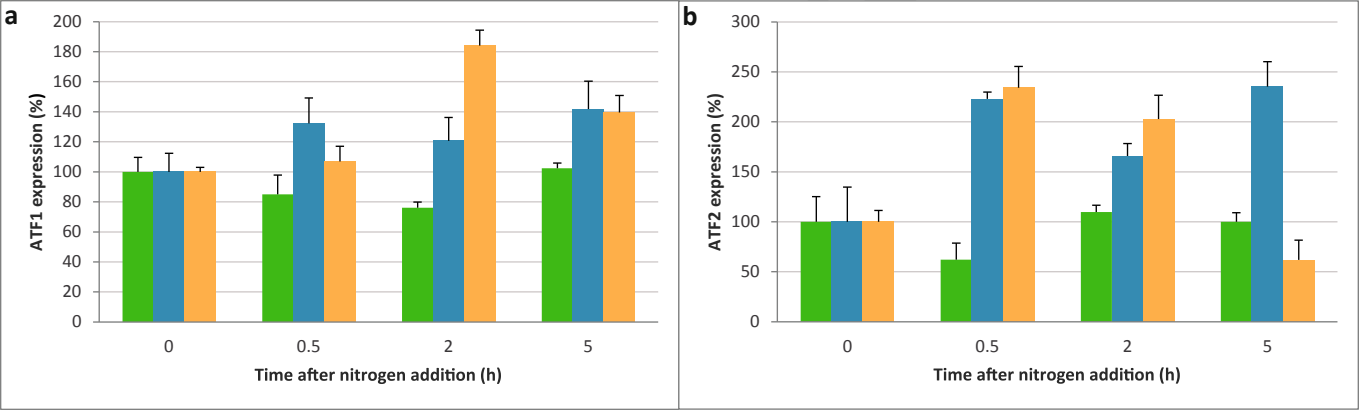

Specific rate of ethyl hexanoate production (mg/L.h)
7E-13
6E-13
5E-13
4E-13
3E-13
2E-13
1E-13
0
0
20
40
60
80
100
120
140
160
180
200
Consumed sugar (g/L)

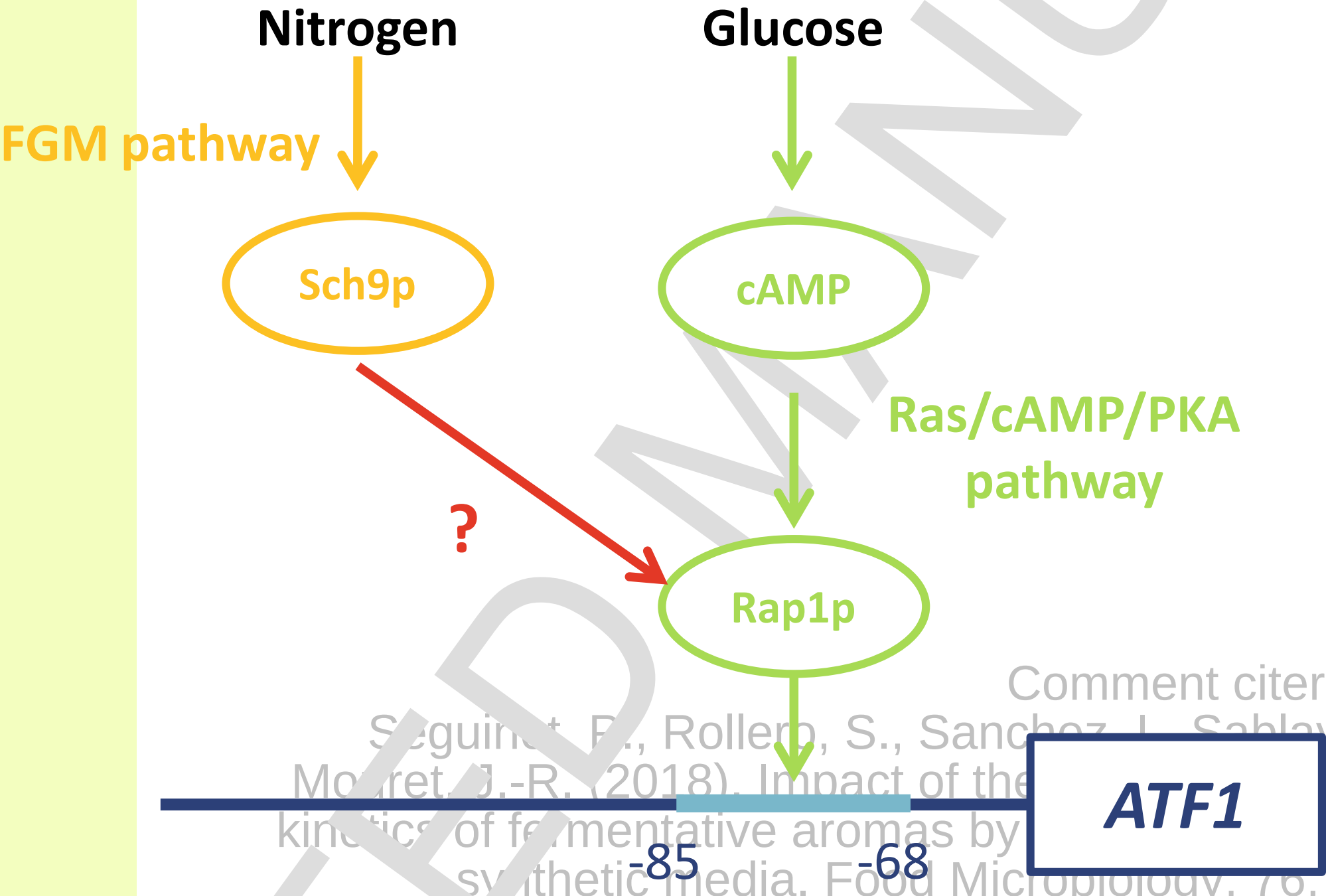

Total production of propanol (mg/L)
Assimilable nitrogen (mgN/L)
Consumed sugar (g/L)
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
160
180





synthetic media. Food Microbiology 76, 2


a
ATF1 expression (%)
200
180
160
140
120
100
80
60
40
20
0
0
0.5
2
5
Time after nitrogen addition (h)
b
ATF2 expression (%)
300
250
200
150
100
50
0
0
0.5
2
5
Time after nitrogen addition (h)



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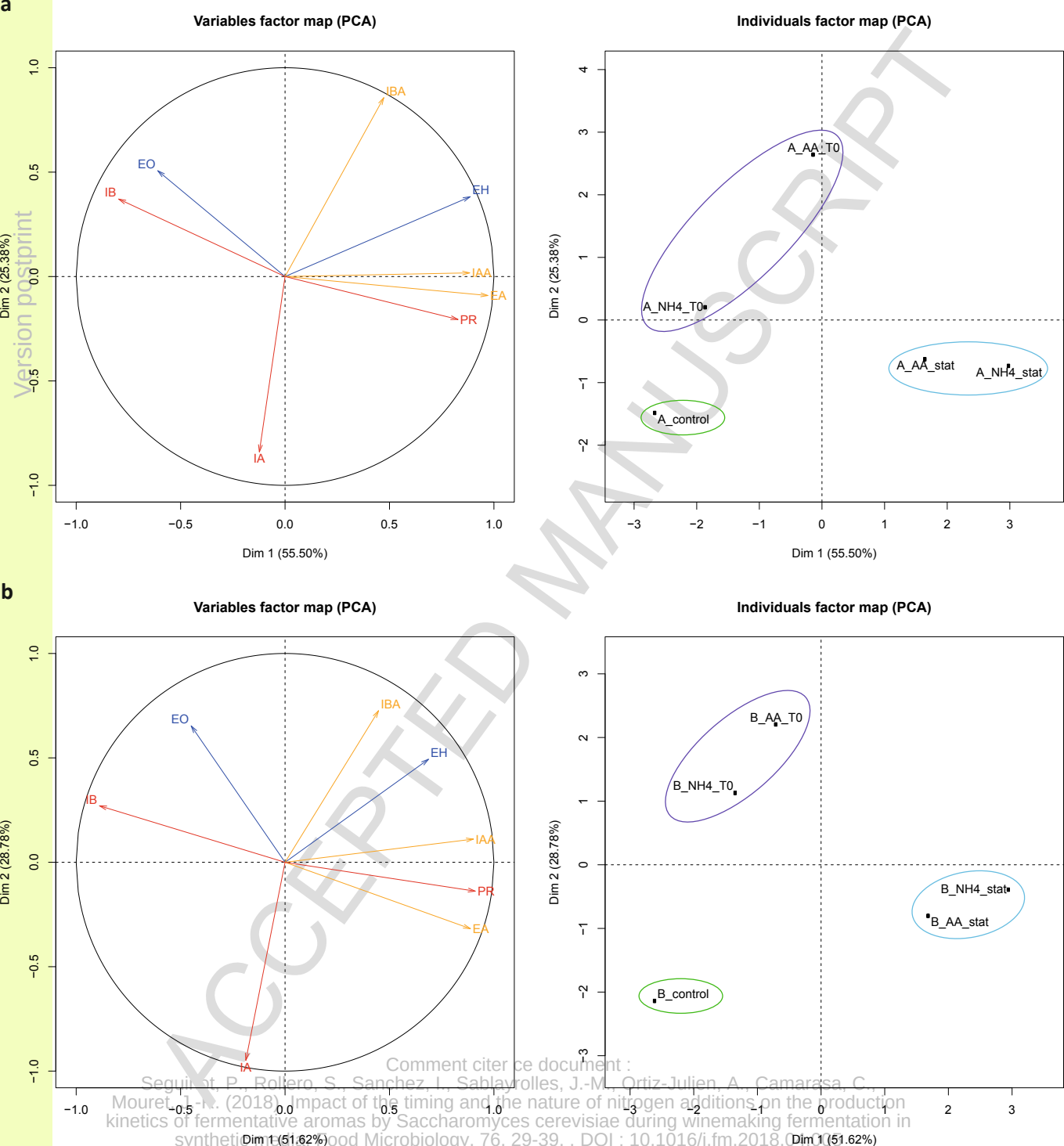

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