Vol. 18, No. 2 (2019) 451-462
Revista Mexicana de Ingeniería Química

THE PRODUCTION OF ESTERS AND GENE EXPRESSION BY *Saccharomyces cerevisiae* DURING FERMENTATION ON *Agave tequilana* JUICE IN CONTINUOUS CULTURES

PRODUCCIÓN DE ÉSTERES Y EXPRESIÓN GÉNICA POR *Saccharomyces cerevisiae* DURANTE LA FERMENTACIÓN DE JUGO DE *Agave tequilana* EN UN PROCESO CONTINUO

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Received: June 13, 2018; Accepted: October 2, 2018

Abstract

Volatile esters are formed during alcoholic fermentation, particularly performed by yeasts. These compounds confer “fruity" attributes to drinks. Fermented beverages contain namely two groups of esters: acetate esters formed by alcohol acetyltransferase enzymes (*ATF1* and *ATF2*) and ethyl esters produced by the acyl-CoA ethanol O-acyltransferase enzymes (*EHT1* and *EEB1*), encoded by the *ATF1*, *ATF2*, *EHT1* and *EEB1* genes. The purpose of this study was to evaluate the effect of nitrogen addition, aeration and low temperatures on ester production during fermentation on *Agave tequilana* juice in a continuous system. Low temperatures increased ethyl esters production (4.5-fold) and enhanced the expression of the involved genes (more than 3.5-fold). Conversely, when compared to aeration, the effect was negative as esters production decreased (75%) whereas the *ATF1*, *ATF2* and *EHT1* genes were repressed. On the other hand, the addition of nitrogen favored the production of ethyl acetate. The results obtained during continuous culture with *Agave tequilana* juice showed that an increase in gene expression favored mainly the production of ethyl esters and not so much the production of ethyl acetate. Therefore, the three genes evaluated do not seem to be the only ones involved in the production of ethyl acetate, they are more related to the production of ethyl esters. So, even though all these compounds belong to the same family, the regulation of their production, as well as their synthesis pathways, seem to be different.

Keywords: *S. cerevisiae*, gene expression, esters, *Agave tequilana*, continuous culture.

Resumen

Durante la fermentación alcohólica los ésteres volátiles son formados principalmente por levaduras, estos compuestos son responsables del carácter afrutado de las bebidas. Existen dos grupos principales de ésteres en las bebidas fermentadas: ésteres de acetato formados por enzimas alcohol acetiltransferasas (*ATF1* y *ATF2*) y ésteres etílicos catalizados por enzimas acil-CoA etanol O-aciltransferasa (*EHT1* y *EEB1*), las cuales son codificadas por los genes *ATF1*, *ATF2*, *EHT1* y *EEB1*. El propósito de este estudio, fue evaluar el efecto de la adición de nitrógeno, aireación y bajas temperaturas en la producción de ésteres durante la fermentación de jugo de *Agave tequilana* en un sistema en continuo. Las bajas temperaturas favorecieron la producción de etíl ésteres (4.5 veces), incrementando los niveles de expresión de los genes involucrados (más de 3.5 veces). En contraste con la aireación, el efecto fue negativo debido a que disminuyó la producción de ésteres (75%) y se observó represión génica de *ATF1*, *ATF2* y *EHT1*. Por otra parte, la adición de nitrógeno favoreció la producción de acetato de etilo. Los resultados obtenidos durante el cultivo en continuo con jugo de *Agave tequilana* demostraron que un incremento en la expresión génica favoreció principalmente la producción de los ésteres etílicos y no tanto la producción del acetato de etilo. Por lo tanto, los tres genes evaluados no parecen ser los únicos involucrados en la producción del acetato de etilo, están más relacionados con la producción de los ésteres etílicos. Entonces, a pesar de que todos estos compuestos pertenecen a la misma familia, la regulación de su producción, así como sus vías de síntesis parecen ser diferentes.

Palabras clave: *S. cerevisiae*, expresión génica, ésteres, *Agave tequilana*, cultivo continuo.

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https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n2/Iniguez
issn-e: 2395-8472

1 Introduction

Esters are synthesized from one alcohol molecule and either an organic or a fatty acid. Yeasts produce the major esters contained in alcoholic beverages by performing an enzymatic esterification of free alcohols and activated carboxylic acids in the form of acyl-CoA (Pires *et al*., 2014). Fermented beverages contain two main groups of flavor-conferring esters. The first group includes acetate esters, such as ethyl acetate, isoamyl acetate and phenyl ethyl acetate. The second group comprises ethyl esters and it includes ethyl hexanoate or ethyl caproate, ethyl octanoate or ethyl caprylate and ethyl decanoate or ethyl caprate (floral odor) (Saerens *et al*., 2008).

Even the slightest concentrations changes of these compounds may highly affect the sensory quality of fermented beverages as their detection thresholds are very low. Considering this, understanding the mechanisms of ester synthesis and how their levels are controlled on the final product is of great interest for the industry. Consequently, the biochemical synthesis of esters has been intensively studied (Van Laere *et al*., 2008).

Different enzymes are involved in ester synthesis. The best characterized are alcohol acetiltransferases I and II (AATase I and II, EC 2.3.1.84) encoded by the *ATF1* and *ATF2* genes, respectively (Fujii *et al*., 1994; Fujii *et al*., 1996; Malcorps and Dufour, 1992; Nagasawa *et al*., 1998; Yoshimoto *et al*., 1999). The formation of ethyl esters in yeast is catalyzed by two acyl-CoA enzymes: *EHT1* and *EEB1* ethanol O-acyltransferases, encoded by the *EHT1* and *EEB1* genes (Saerens *et al*., 2006). The identification of genes and enzymes responsible for ester synthesis enabled the elucidation of the molecular factors controlling the rate of ester synthesis (Van Laere *et al*., 2008).

In *S. cerevisiae*, ethyl acetate and isoamyl acetate production is controlled by overexpression of *ATF1* and *ATF2* respectively, in contrast to what is *ATF2* playing a minor role compared to *ATF1* (Lilly *et al*., 2006; Verstrepen *et al*., 2003b). However, if the *ATF1* and *ATF2* genes are deleted, significant amounts of ethyl acetate and isobutyl acetate are still produced. This provides additional evidence for the existence of a yet unknown ester synthases within the yeast proteome (Verstrepen *et al*., 2003b). Recently, a new enzyme (*EAT1*) was discovered in the yeast *Wickerhamomyces anomalus*. A high ethyl acetate production was observed when this enzyme was expressed in either *Saccharomyces cerevisiae* or *Escherichia coli* (Kruis *et al*., 2017). The overexpression of *EHT1* results in a marked increase in ethyl caproate, ethyl caprylate, and ethyl caprate (Lilly *et al*., 2006).

Basically, there are two main factors involved in ester formation: 1) the concentration of both substrates: acyl-CoA and the respective alcohols, and 2) the overall activity of the enzymes involved in their synthesis and breakdown. Thus, all parameters affecting substrate levels or enzyme activity have an impact on ester production (Verstrepen *et al*., 2003). The external factors are: culture medium composition (particularly the carbon / nitrogen ratio), pH, temperature, the addition of fatty acids, nitrogen and oxygen levels, among others.

In *S. cerevisiae*, nitrogen source and its concentration impacts on ester production. Hernández-Orte *et al*. (2005) suggested that the amino acid composition may be important to establish the flavor profile. Arrizon and Gschaedler (2007) observed that volatile compounds synthesis depends on the nitrogen source added. Additionally, it was found that branched chain fatty acids and their esters occur with low nitrogen concentrations, whereas medium-chain fatty esters and acetic acid were correlated with high nitrogen levels (Vilanova *et al*., 2007).

Yeasts grown with a suitable aeration synthesize ethanol and other alcohols, but ester formation is suppressed in these conditions (Berry and Watson, 1987). Oxygen promotes yeast growth and the concomitant use of acetyl-CoA. In such conditions, the latter species is depleted thus restricting ester production (Verstrepen *et al*., 2003). After cloning the *AAT* genes *ATF1* and *ATF2*, it was shown that gene transcription mediated by the former is directly repressed by both unsaturated fatty acids and oxygen (Fujii *et al*., 1997; Fujiwara *et al*., 1999).

Although it is expected that low temperatures increase the amount of esters during fermentation, the experimental evidence previously published did not support this hypothesis, leaving a rather unclear landscape. Some studies described that ester production is stimulated at low fermentation temperatures (Erten, 2002). However, other reports showed lower ester levels as consequence of a low fermentation temperature (Llaurado *et al*., 2002). Conversely, ester production increased at higher temperature (Saerens *et al*., 2008). Molina *et al*., (2007) reported that lower temperatures increase the production of volatile compounds and this effect may

be caused by differential expression of the *ATF1*, *ATF2*, IAH1 and *EHT1* genes. These are all involved in aromatic attributes of *S. cerevisiae*.

Reproducible and reliable data is needed in order to conduct transcriptional studies in several conditions by using techniques such as qPCR, microarrays, differential transcriptome analysis, among others (Hoskisson and Hobbs, 2005). Data acquisition for biology systems studies is essential to conduct experiments in controlled and reproducible conditions. For example, the use of continuous culture to grow microorganisms is an ideal tool to achieve these objectives (Winder and Lanthaler, 2011). This system is useful to study microbial growth and its physiology when compared to heterogeneous batch cultures as the induced stress and the dynamic physical-chemistryl conditions in the latter system yield complex data difficult for interpretation purposes (Hoskisson and Hobbs, 2005). Most of the transcriptome studies published to date on *S. cerevisiae* have been carried out in batch cultures. In these conditions, the specific growth rate (μ) is affected by experimental conditions. This is a relevant matter as the specific growth rate per se has a strong impact on the genome transcript profiles (Castrillo *et al*., 2007; Regenberg *et al*., 2006; Tai *et al*., 2007). However, the use of a continuous system to study physiology during the fermentative stage is not common (Morán-Marroquín *et al*., 2011; Vázquez-Lima *et al*., 2014) and a few number of studies have been conducted during cell growth (Aceituno *et al*., 2012; Tai *et al*., 2007). The aim of this work was to evaluate the effect of nitrogen, aeration and low temperatures on ester production as well as the expression of the genes involved in their synthesis during continuous fermentations on *Agave tequilana* juice.

2 Materials and methods

2.1 Yeast strain

The *Saccharomyces cerevisiae* AR5 strain was used. The latter was isolated from *Agave tequilana* Weber juice fermentation and it was obtained from the CIATEJ culture collection (Research Center in Technology and Design Assistance of Jalisco State, México).

2.2 Culture media

The filtered juice from *Agave tequilana* Weber var. azul was provided by "La Madrileña S.A. de C.V.", a tequila distillery (Jalisco, México). The concentration of reducing sugars contained in the agave juice was adjusted to 100 g/L. The culture media was supplemented with ammonium sulfate (0.5 g/L) and it was sterilized at 121 ºC for 15 min.

2.3 Inoculum preparation and fermentation conditions

Initial cultures were grown in 50 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) for 24 h at 30 ºC and 250 rpm shaking. Continuous culture was carried out in a 3 L bioreactor (Applikon, The Netherlands) using a 1.5 L working volume. To obtain online data, the bioreactor was equipped with pH and dissolved oxygen sensors (Applikon) connected to an Applikon AD1030 biocontroller on a computer installed with the Bioexpert software v.1.50.114. Exhaust gases were monitored by using O_2 and CO_2 sensors (Bluesens, Herten, Germany) and data was acquired using the Bacvis software v.7.6.2.1. Cultures were initiated in batch mode, by inoculating fermentation medium containing 1×10^6 cells/mL and afterwards incubating at 30 ºC and 250 rpm for 12 h. Sterilized fermentation medium was fed to the culture. The initial medium feed rate and other fermentation parameters are shown in Table 1. Two peristaltic pumps (Cole-Parmer, Barrington, IL, USA) were synchronized to simultaneously feed fresh medium and to extract fermented medium. During the aeration stage, air was injected through a sterile HepaVENTTM filter into the bioreactor at 0.5 vvm.

A continuous culture was carried out and samples were taken along it.

Table 1. Parameters of the continuous fermentation process.

Fermentation parameters	*S. cerevisiae* (AR5)
Flow (mL/min)	4.46
Specific growth rate (μ) (h^{-1})	0.18
Agitation (rpm)	250.00
Aireation (vvm)	0.50
Nitrogen (ammonium sulfate) (g/L)	1.00
5 residence times (h)	28.00
Fermentation time (h)	180.00

vvm=Gas-volume flow/unit of liquid volume/minute.

Table 2. Sampling description.

Key	Condition	*S. cerevisiae* (AR5) Sequential Time (h)
B	Basal	39.5
N1	Nitrogen after 0.5 h	40.5
N2	Nitrogen after 8 h	48
N3	Nitrogen after 27.5 h	67.5
A1	Aeration after 0.5 h	68.5
A2	Aeration after 4 h	72
A3	Aeration after 27.5 h	95.5
30°	30 ºC after 27.5 h	123.5
25°1	25 ºC after 0.5 h	124.5
25°2	25 ºC after 6 h	130
25°3	25 ºC after 27.5 h	151.5
20°1	20 ºC after 0.5 h	152.5
20°2	20 ºC after 21.5 h	173.5

In order to attain the steady state in each studied condition, the culture was maintained for five residence times and samples were collected in each condition. Table 2 shows the sampling times. 5-mL samples of culture medium were taken from the bioreactor throughout the fermentation to assess cell number and to analyze the supernatant (volatile fermentation products). Supernatant samples were stored at -20 ºC before use. For RNA isolation, 10-mL samples were collected from each of culture medium and immediately stored at -80 ºC.

The experimental conditions include nitrogen addition, aeration and temperature drops at 25 ºC and 20 ºC. When nitrogen was added, ammonium sulfate concentration increased from 0.5g/L to 1 g/L.

2.4 *Analytical methods*

Sample population was quantified under a microscope by using a Neubauer chamber. Reducing sugars were assessed according to the technique established by Miller (1959). The volatile compounds produced during fermentation were quantified by gas chromatography. A Hewlett Packard Head-space 7694E connected to a HP 6890 Series gas chromatograph equipped with a flame ionization detector (FID) and a HP-INNOWAX column (60 m $\times$ 0.32 mm $\times$ 0.25 μm) was used. Helium was used as a gas carrier. The oven temperature program was set to 45 ºC for 7 min, then it was increased to 160 ºC at a 10 ºC/min rate and finally it was increased to 220 ºC at a 20 ºC/min rate. The FID detector temperature was 250 ºC. Vial oven, loop and transfer line temperatures were 80, 110 and 115 ºC, respectively. Heating Ttimes, pressurization and withdrawal were set to 40, 0.2 and 0.2 min, respectively. The vial equilibration time and sample agitation were 0.5 and 1 min respectively.

2.5 *mRNA Extraction and cDNA synthesis*

The isolation of total nucleic acids (TNA) was carried out by using the Masterpure™ Yeast DNA Purification kit (Epicentre an Illumina® company, Madison,WI, USA) as recommended by the manufacturer. 0.5 μL of DNase I (375 U/μL, Invitrogen™, Carlsbad, CA, USA) was added to tubes containing 50 μL of TE buffer and TNA. They were subsequently mixed and incubated for 40 min at 37 ºC. mRNA was isolated by using the GenElute™- mRNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) as recommended by the manufacturer. RNA concentration was measured by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Reverse transcription was performed by using 8 μL of total mRNA as template with oligo-dT primers.and the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™, Carlsbad, CA, USA) as recommended by the manufacturer.

2.6 *Relative gene expression by real time-PCR*

The primers for the *S. cerevisiae EHT1* gene (F 5'-CACGTTCCAAAATTACCACCA-3'; R 5'-TATCGCCCTCTTCTCCCA-3') were designed using the automatic function of CLC Main Workbench 5.5. The *S. cerevisiae ATF1* and *ATF2* genes were sequences previously reported by Molina *et al*. (2007).

Primers for the ACT1 (actin) (Babiskin and Smolke, 2011), *QCR9* (Cytochrome b-c1 complex subunit 9) (Vaudano *et al*., 2009) and *TFC1* genes (Transcription factor tau 95 kDa subunit) (Teste *et al*., 2009) were included as reference genes.

Real time-PCR (qPCR) reactions were performed as triplicate in a LightCycler 1.5 (Roche Diagnostics GmbH, Mannheim, Germany). The qPCR reaction contained 2 μL of LightCycler® FastStart DNA MasterPLUS SYBR Green I 2X (Roche Applied Science, Mannheim, Germany), 0.2 μL of each primer (10 μM), 63-71 ng of cDNA template. The final volume was adjusted to 10 μL. PCR program conditions were 95 ºC for 10 min, 40 denaturation cycles at 95 ºC for 20 s, the annealing step ranged from 48 to 58 ºC for 10 s and an extension was performed at 72 ºC for 20 s. The melting curve program was 95 ºC for 5 s and 65 ºC for 15 s. At least two negative controls were used per run including the non-template control and the RT-negative control. The relative quantitative gene expression was evaluated according to the method reported by Pfaffl (2001). The "basal" sample is equivalent to the "control sample" concept in the Pfaffl formula. The control group mean value was normalized to 1. Relative expression was normalized to the cell number, RNA and cDNA inputs.

2.7 *Statistical analysis*

F tests were performed in order to assess if intergroup variances were different or equal, followed by *t* tests to compare the mean value of fold changes among the several conditions and the "basal" sample. The correlation between gene expression and ester concentration was evaluated by using Minitab v.16 (State College, Pennsylvania, U.S.). A *p-value* less than 0.05 was considered statistically significant.

3 Results and discussion

3.1 *Continuous culture for transcriptional analysis*

In this study we used a continuous culture to evaluate the effect of nitrogen addition, aeration and low temperatures on the production of volatile compounds and the expression of the genes involved in the ester synthesis with *Agave tequilana* juice used as substrate.

Figure 1 shows cell population, the concentration of reducing sugars and overall ethanol production throughout the fermentation process.

An initial batch culture was performed for 12 hours and subsequently a continuous cultivation was started by feeding the reactor at a 4.46 mL/min flow rate to attain a specific growth rate of 0.18 h^{-1} (dilution rate, D) defined as an approximation to the μ_{max} (0.46 h^{-1} ± 0.02). Each study condition was maintained for five residence times and it is represented by vertical dotted lines in Figure 1.

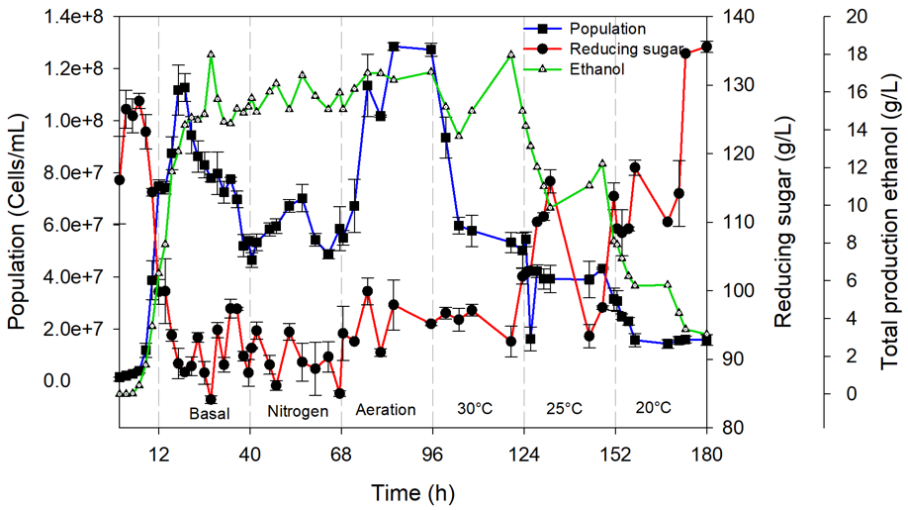

Fig. 1. Yeast population, reducing sugar and ethanol production during continuous culture of *S. cerevisiae* (AR5) yeast fed with *Agave tequilana* juice at different fermentation conditions.

The basal condition (12-40 h) was used as a control for comparison purposes. The cell population increased by 40% during the first hours after the pulse of nitrogen (40 h). Once the added nitrogen consumed, the population returned tothe values observed before the pulse. The maximum cell concentration was reached under aeration conditions (1.28×10^8 cells/mL).

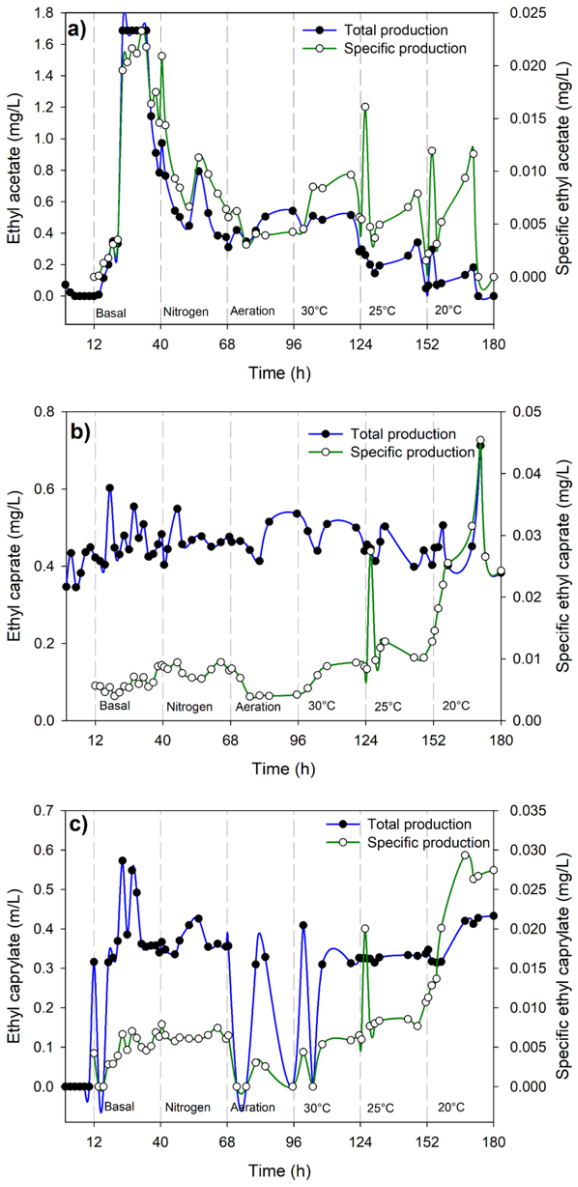

Fig. 2. Total and specific production (normalized to 1×10^6 cells) of a) ethyl acetate, b) ethyl caprate and c) ethyl caprylate by *S. cerevisiae* (AR5) during continuous culture fed with *Agave tequilana* juice at different fermentation conditions.

However, the reducing sugar level did not decrease, suggesting that a high sugar consumption is not necessary to attain a high cell population (68-96 h).

After aeration, five residence times were maintained at 30 ºC (96-124 h) in which substrate consumption rate was constant, although cell population decreased. The ethanol production ranged between 14 and 17 g/L in basal condition and at 30 ºC (20-124 h) respectively (see Figure 1).

At 25 ºC (124-152 h) cell population initially decreased and reducing sugars accumulated. However, after the first hours the cell population was recovered, whereas the reducing sugars were consumed up to a 95 g/L value. At the end, ethanol concentration decreased to 8 g/L. Finally, at 20 ºC (152-180 h), cell population decreased to 1.5×10^7 cells/mL (90%) and a constant value was reached after 158 h; whereas reducing sugars accumulated until a 136 g/L concentration. Ethanol concentration decreased to 3 g/L as low temperatures induce a decreasing of the metabolic activity of the organism, therefore, the specific growth rate at low temperatures decreases accordingly (see Figure 1).

Generally, the continuous cultivation showed a stable behavior and after nitrogen addition under aeration it returned to the basal state (30 ºC). After five residence times it was observed that cell population and ethanol production were similar regardless of the specific condition (Figure 1). Additionally, CO_2 and O_2 percentages were similar and they remained constant in both conditions (data not shown). This demonstrates the usefulness of continuous cultures to study *S. cerevisiae* in different physiological conditions.

Samples were taken 30 minutes after the modification of the condition d (N1, A1, 25°1, 20°1) and after pH and CO_2 values as well as cell population were stabilized (N2, A2, 25°2, 20°2), and, finally, 30 minutes before switching to another condition (N3, A3, 25°3) (see Table 2). Samples collected during the continuous fermentation were analyzed for volatile compound levels and the expression of *ATF1*, *ATF2* and *EHT1* genes. In this study, the ACT1 gene was selected as reference as it was more stable during the different experimental conditions when compared to *QCR9* and *TFC1* (data not shown). It is important to specify that the total production of volatile compounds is the quantity of volatile compounds measured in each time of fermentation.

Table 3. Specific production of volatile compounds normalized to 1×10^6 cells during continuous culture of *S. cerevisiae* (AR5) yeast fed with *Agave tequilana* juice at different fermentation conditions.

Condition	Ethanol (g/L)	Ethyl acetate (mg/L)	Ethyl caprate (mg/L)	Ethyl caprylate (mg/L)
B	0.283	0.015	0.009	0.006
N1	0.337	0.021	0.009	0.008
N2	0.276	0.008	0.008	0.006
N3	0.273	0.006	0.008	0.006
A1	0.274	0.006	0.008	0.006
A2	0.24	0.006	0.007	ND
A3	0.134	0.004	0.004	ND
30°	0.298	0.006	0.009	0.006
25° 1	0.26	0.005	0.008	0.006
25° 2	0.28	0.004	0.012	0.008
25° 3	0.257	0.002	0.013	0.011
20° 1	0.257	0.002	0.015	0.011
20° 2	0.214	ND[a]	0.027	0.027

[a]Not detected

On the other hand, the specific production is the relation between the total production and the population present in each fermentation time; the specific production allows to observe the production of volatile compounds for each million cells.

Table 3 shows specific production of both ethanol and esters in conditions where gene expression was quantified. Compound concentration was normalized to 1×10^6 cells in order to compare specific production values in each condition. Total and specific ester production throughout the continuous culture is show in figure 2. It may be noticed that specific production of both esters and ethanol is affected by all the studied factors.

3.2 Effect of the nitrogen concentration on ethanol and ester production as well as gene expression

The addition of ammonium sulfate as nitrogen source was tested during the continuous fermentation by *S. cerevisiae*. The obtained results show that, although no significant change of overall ethanol production was observed in this condition (Figure 1), its specific production increased (Table 3). This is consistent with the results obtained by Arrizon and Gschaedler (2007) and De los Rios-Deras *et al*., (2015) . The highest ethanol production was obtained 30 minutes after the addition of ammonium sulfate (N1) and it reached 0.337 g/L$\times10^6$ cells. When the maximum concentration of nitrogen source was tested (1 g/L), the activity of sugar transporters increased concomitantly causing an accelerated fermentation rate and enhancing ethanol production. Nitrogen addition also caused an increase of ethyl acetate and ethyl caprylate production (from 0.015 to 0.021 and from 0.006 to 0.008 mg/L$\times10^6$ cells, respectively), as reported by Vilanova *et al*., (2007) during a fermentation on a synthetic medium similar to grape juice and De los Rios-Deras *et al*., (2015) during fermentation of *A. duranguensis* must was enhanced by adding 50% more nitrogen (Table 3).

The metabolic changes triggered in each condition may result in the activation and/or deactivation of some pathways leading to ester formation. Nitrogen addition downregulated ($p < 0.05 - 0.01$) three of the *S. cerevisiae* genes evaluated in this study (see Figures 3a, 3b and 3c). However, no report is available regarding *ATF1*, *ATF2* and *EHT1* gene expression after nitrogen addition, so this is the first time that the effect of downregulation of ester synthesis is reported in *S. cerevisiae*

3.3 Aeration effect on volatile compound production and gene expression

Some significant effects were observed under aeration. First, total ethyl acetate production decreased regarding the basal condition. This is in agreement with the results reported by other researchers as they point out that aeration decreases ester production (Fujii *et al*., 1997). Regarding ethyl caprylate production, it remained constant under aeration when compared to the basal condition, and even the

production of ethyl caprate increased 35% regarding the basal values (0.40 to 0.54 mg/L). However, the specific production of these esters decreased. This demonstrates that the ester production per million yeasts decreases under aeration when compared to the basal condition. Nevertheless, when population increases, this specific production may be equal and even higher than the basal production (Table 3 and Figure 2).

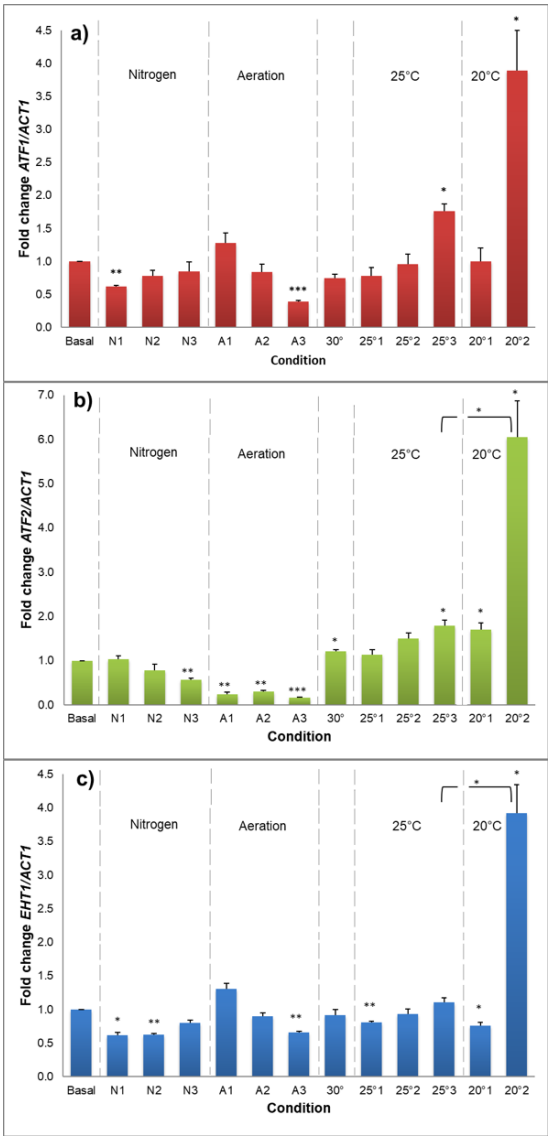

Fig. 3. Relative expression of a) *ATF1* gene, b) *ATF2* gene and c) *EHT1* gene by *S. cerevisiae* (AR5) during continuous culture fed with *Agave tequilana* juice at different fermentation conditions. *p 0.05 – 0.01, **p 0.01 – 0.001, ***$p < 0.001$.

These results are consistent with those previously reported by Plata *et al.* (2005).They demonstrated that aerobic conditions caused a decreasing of the production of acetate esters in wine. Valero *et al.* (2002) also concluded that the optimal values for higher alcohol and ester production are attained in the absence of oxygen. Both studies reported the specific production of volatile compounds.

Previous studies carried out on continuous fermentations by *S. cerevisiae* strains on agave juice (Hernández-Cortés *et al.*, 2010; Morán-Marroquín *et al.*, 2011), concluded that aeration increased both biomass production and reducing sugars consumption. Morán-Marroquín *et al.* (2011) also observed that micro-aeration increased ethanol and volatile compound productions, although only total production is mentioned. However, it is known the aerobic metabolism decreased acetate esters levels, probably as a consequence of the accelerated yeast growth. This effect was also observed on batch fermentations by a *S. cerevisiae* strain on grape juice (Fujii *et al.*, 1997). It has been reported that the *ATF1* gene is repressed by aeration and it affects *ATF1* mRNA levels, thus decreasing AATase activity (Fujii *et al.*, 1997). The obtained results in this study showed the higher down-regulation of all three evaluated genes, in the case of *ATF1* and *EHT1* were repressed until the last sample of the aeration condition (A3) ($p < 0.001$ for *ATF1*, $p < 0.01$ for *EHT1* and $p < 0.001$ for *ATF2*) (Figure 3). This may be caused by a limitation of the available acetyl-Co-A used to increase yeast cell density during aerobic fermentation. Consequently, this restricts the production of esters as such intermediate function as their precursor (Nordström, 1963).

3.4 The effect of low temperatures on volatile compound production and gene expression

It is known that low fermentation temperatures induce metabolism changes that yields secondary metabolites, including aroma-conferring compounds such as esters. Low temperatures are common during wine production in order to enhance fruitiness and tropical attributes (Mallouchos *et al.*, 2007; Molina *et al.*, 2007). In this study, the highest concentration of ethyl esters is reached at 20 ºC. The specific production of ethyl caprate was 0.045 mg/L×10^6 cells, whereas that of ethyl caprylate was 0.03 mg/L×10^6 cells (Figures 2b and 2c, respectively).

Table 4. Statistical analysis and correlation between esters concentration and gene expression levels.

Genes	*S. cerevisiae* (AR5)					
	ATF1		*ATF2*		*EHT1*	
Esters	*p-value*	*r*	*p-value*	*R*	*p-value*	*r*
Ethyl acetate	0.125	-0.448	0.198	-0.382	0.168	-0.407
Ethyl caprate	4.40×10^{-6}	0.929	4.78×10^{-8}	0.969	6.02×10^{-5}	0.884
Ethyl caprylate	1.36×10^{-5}	0.913	2.85×10^{-7}	0.957	1.35×10^{-4}	0.865

However, the maximum ethyl acetate production occurred in basal conditions and low levels were observed throughout the rest of the fermentation process. Nevertheless, an increased in the specific production of ethyl acetate was observed when temperature was decreased to 25 ºC and 20 ºC. Such ester production increase may be the consequence of a stress response displayed by the yeast (Table 3 and Figure 2). In contrast, these results do not support those obtained by Saerens *et al*., (2008) as they reported a higher ethyl caproate and caprylate production at higher temperatures by using the industrial *S. cerevisiae* CMBS SS01 strain. However, different operating conditions and strains can play an important role for the obtained outcomes.

In this study, the *ATF1* and *ATF2* genes were significantly upregulated when compared to the basal condition ($p < 0.05$) at 25 ºC after 27.5 h. Moreover, the overexpression of all the studied genes was observed ($p < 0.05$), after 21 h at 20 ºC (Figure 3). These results support the conclusion established by Molina *et al*., (2007), as they mention that lower temperatures increased volatile compound production and this effect may be caused by the differential expression levels of *ATF1*, *ATF2*, IAH1 and *EHT1*, all of them involved in aromatic compounds pathways in *S. cerevisiae*. The physiological role for ester production by yeasts is still unknown, Christiaens *et al*., (2014) suggested that acetate esters seem to attract fruit flies that function as vectors in order to promote yeast dispersal. They observed that *ATF1* deletion in yeast drastically reduces *Drosophila* attraction and therefore it limits their dispersal. Thus, it is possible that yeast produce esters when submitted to stress conditions by low temperatures.

3.5 Correlation between gene expression levels and ester concentration

For each condition, the expression level of the *ATF1*, *ATF2* and *EHT1* genes was correlated with the measured ester levels. A statistically significant correlation was established ($p < 0.001$) between gene expression and both ethyl caprylate and ethyl caprate levels. The correlation coefficient was r^2 = 0.884-0.969 and the most significant correlation was observed with the *ATF2* gene (Table 4).

Nevertheless, it has been reported that only *EHT1* and *EEB1* genes are involved in ethyl ester synthesis (Saerens *et al*., 2006). Interestingly, no association was found between ethyl acetate and the studied genes, despite the findings by Verstrepen, *et al*. (2003b). These authors report that *ATF1* and *ATF2* expression levels impact on acetate esters production in *S. cerevisiae*. However, they also found additional evidence on others ester synthases that are remain to be identified on the yeast proteome. After deleting the *ATF1* and *ATF2* genes, their strain still produced 50% of the overall ethyl acetate in spite the suppression of these genes. The discovery of the Eat1enzyme by Kruis *et al*. (2017), confirms the existence of other enzymes involved in esters synthesis, as previously proposed by Verstrepen, *et al*. (2003b). In this study it was observed that, although the *ATF1* and *ATF2* genes are homologous, they are different and consequently their expression levels in all conditions also differed.

Conclusions

In this study, the effect of different fermentation conditions on volatile compound production by the yeast *S. cerevisiae* (strain AR5) and on the expression levels of selected genes was shown by performing a continuous fermentation on *Agave tequilana* juice. Lower temperatures enhanced ethyl esters production. The latter are associated with a sour apple and fruity aroma. However, aeration significantly decreased esters production. Additionally, nitrogen addition induced a substantial increase of both ethanol and ethyl acetate production. Additionally, this study provides evidence that esters production may derive from differential expression of the genes involved in aroma pathways (*ATF1*, *ATF2*, *EHT1*) as a high correlation was observed between their expression

levels and the concentration of ethyl esters. However, no correlation was found between ethyl acetate production and the *ATF1* and *ATF2* genes. A possible explanation is that the recently reported *EAT1* enzyme or another yet undiscovered enzymes play a more prominent role to produce this compound. This work demonstrates that low fermentation temperatures and no aeration may be used to increase the concentration of esters during continuous fermentation on *Agave tequilana* juice. These results are relevant for tequila and mezcal manufacturers looking to obtain products with enhanced aromatic attributes.

Acknowledgements

This work was supported by Jalisco's Mixed Fund (FOMIX JALISCO) as part of the project "Study of the synthesis of esters in the fermentation process of tequila: strategies to promote the synthesis of volatile compounds" (Number 123157) and by Mexico's National Council for Science and Technology (CONACYT). Laura Elena Iñiguez Muñoz is a CONACYT doctoral fellow.

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