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ORIGINAL ARTICLE

Effects of the addition of different nitrogen sources in the tequila fermentation process at high sugar concentration

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Abstract

Aims: To study the effect of the addition of different nitrogen sources at high sugar concentration in the tequila fermentation process.

Methods and Results: Fermentations were performed at high sugar concentration (170 g l^{-1}) using *Agave tequilana* Weber blue variety with and without added nitrogen from different sources (ammonium sulfate; glutamic acid; a mixture of ammonium sulfate and amino acids) during the exponential phase of growth. All the additions increased the fermentation rate and alcohol efficiency. The level of synthesis of volatile compounds depended on the source added. The concentration of amyl alcohols and isobutanol were decreased while propanol and acetaldehyde concentration increased.

Conclusions: The most efficient nitrogen sources for fermentation rate were ammonium sulfate and the mixture of ammonium sulfate and amino acids. The level of volatile compounds produced depended upon types of nitrogen. The synthesis of some volatile compounds increased while others decreased with nitrogen addition.

Significance and Impact of the Study: The addition of nitrogen could be a strategy for improving the fermentation rate and efficiency in the tequila fermentation process at high sugar *Agave tequilana* concentration. Furthermore, the sensory quality of the final product may change because the synthesis of the volatile compounds is modified.

Introduction

'Tequila' is a Mexican alcoholic beverage obtained by distilling and rectifying fermented agave juice and is produced in a territory protected by a guarantee of origin. There are two main types of tequila: tequila 100% obtained exclusively from sugars of the *Agave tequilana* Weber blue variety and tequila produced using 51% of sugars from *A. tequilana* Weber blue variety and 49% sugars from other sources like sugar cane, 'sugar-loaf', molasses, or hydrolysed corn syrup. The process is divided into four main phases: cooking, milling, fermenting and distilling. The agave is harvested 8 years after planting and contains an average of 27% reducing sugars (Cedeño 1995). The agaves are cooked in autoclaves or brick ovens. Most of the assimilable nitrogen is degraded by Maillard reactions because of heating (Mancilla-Margalli and Lopez 2002), making the juice poor in available nitrogen content. Normally, for tequila fermentation, the agave juice is diluted with water to reach 12–14°Brix degrees (80–100 g l^{-1} of agave sugar) after milling, this condition corresponds to industrial agave sugar fermentation (IASF). In some cases, an inorganic nitrogen source is added at the beginning of fermentation. It has been found that when tequila is produced at high *A. tequilana* sugar fermentation (HASF), the addition of a mixture of ammonium sulfate and amino acids at the exponential phase of growth, caused an increase in the fermentation rate and alcohol efficiency (Arrizon and Gschaedler 2002). A similar behaviour has been observed in wine fermentations at high sugar concentration with additions of ammonium (Mendes-Ferreira *et al.* 2004) or ammonium and amino acids (Beltran *et al.* 2005). These additions have an impact on volatile compound production. When additions were performed in the early steps of fermentation, a decrease in higher alcohols and an increase in acetaldehyde and ethyl acetate were observed (Beltran *et al.* 2005). In case of tequila, no studies have been performed about the impact of the addition of nitrogen at high sugar concentration on volatile compound production. In this study, a comparison of the fermentation behaviour at HASF conditions and volatile compound production, was evaluated with the addition of nitrogen in the exponential phase of growth, using nitrogen sources of different nature: inorganic nitrogen (ammonium sulfate), organic nitrogen (glutamic acid) and a mixture of inorganic and organic nitrogen (ammonium sulfate and amino acids). Finally, the concentrations of ethanol, methanol, higher alcohols, ethyl acetate and acetaldehyde, produced from fermentations with additional nitrogen sources were compared with the concentrations allowed by the Mexican Tequila standard (NOM-006-SCFI-2005, 2006).

Materials and methods

Yeast strain

The strain of *Saccharomyces cerevisiae* (MG) used in this study was isolated from the tequila industry and conserved in our laboratory collection (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C.).

Culture media

The must used is an *A. tequilana* Weber blue variety juice. It was filtered, sterilized (121°C, 15 min), and diluted with distilled water to reach sugar concentrations of 170 and 80 g l^{-1} (high and normal industrial sugar concentration respectively). In all cases, 1 g l^{-1} of ammonium sulfate was added at the beginning of fermentation. Nitrogen addition in the exponential phase of growth (6 h of fermentation) was performed at high sugar *A. tequilana* concentration (170 g l^{-1}) and was compared with fermentations without nitrogen addition (170 and 80 g l^{-1}). The composition of the nitrogen sources added was: 416·55 mg l^{-1} of ammonium sulfate, 928·60 mg l^{-1} of glutamic acid and 437·12 mg l^{-1} of a mixture of ammonium sulfate and amino acids (alanine, arginine, aspartate, glutamate, glutamine, leucine, phenylalanine, proline, tryptophan and valine). All of them equivalent to 88·36 mg of total assimilable nitrogen. The composition of the mixture of amino acids used was described in a previous work (Arrizon and Gschaedler 2002).

Inoculation conditions

Cells were grown for 12 h at 30°C with shaking (250 rev min^{-1}) in 500-ml Erlenmeyer flasks with 200 ml of must, reaching cell populations of 200×10^6 cells ml^{-1}. The must consisted of *A. tequilana* Weber blue variety juice (60 g l^{-1}) that was filtered and sterilized (121°C, 15 min). The reactor was inoculated with 200 ml, which provided an initial population in the reactor of 20×10^6 cells ml^{-1}.

Fermentation conditions

Batch cultures were carried out under semi-anaerobic conditions in 3-l fermentors containing 2 l of must. Fermentation was run with constant low stirring (200 rev min^{-1}) at 35°C. The medium was saturated with sterilized air before inoculation. The nitrogen addition from each of the different sources was carried out after 6 h of culture at 170 g l^{-1} of agave sugar. Each fermentation was run in triplicate to statistically analyse the volatile compounds and fermentation kinetics.

Monitoring of fermentation

Samples were taken every 2 h during the first 16 h of fermentation and every 4 h until a total of 72 h of culture to determine yeast biomass and the concentrations of sugar, ethanol and volatile compounds.

Analysis

Biomass
Yeast biomass was determined by measuring dry weight. The cellular dry weight was obtained by harvesting the cells from 12 ml of the culture medium by centrifugation (3000 ***g***, 20°C, 20 min), rinsed with the same amount of distilled water, and desiccated at 108°C until constant weight was obtained.

Reducing sugars
The reducing sugar concentration in the medium was determined using 3,5-dinitrosalicylic acid reagent (Miller 1959).

Ethanol
Samples were first distilled, and the resultant ethanol concentration was measured using a dichromate reagent (Boehringer and Jacob 1964).

Volatile compounds

The fermentation samples were distilled and the analysis of volatile compounds was carried out in a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA, USA) with a flame ionization detector equipped with an HP-Innowax PEG column (60 m × 320 μm^2). The column was conditioned at 50°C which was maintained for 6 min, then increased at the rate of 10°C per min to 160°C which was followed by a more rapid increase of 20°C min^{-1} until it reached 220°C. Injector and detector temperature was maintained at 250°C. Sample size was 1 μl and the carrier gas was nitrogen.

Parameter calculation

Alcohol efficiency was calculated from the ratio of the average ethanol produced at the end of fermentation and theoretical ethanol production from the biochemical conversion of the sugar consumed. For maximum specific ethanol and biomass formation or sugar consumption rates, experimental data of ethanol, sugar and biomass concentrations were adjusted to a mathematical model using the program Curve Expert 1·3 (EBT Comm, Columbus, MS, USA). This model was further interpolated (100 points), and the maximum specific rates were obtained from the maximum slopes (sugar, biomass or ethanol divided by biomass produced at each interpolated point and time).

Statistical analysis

Five treatments were applied. Two treatments which consist of IASF and HASF without nitrogen addition and three treatments at HASF conditions with different nitrogen sources added (ammonium sulfate; glutamic acid; a mixture of ammonium sulfate and amino acids). The variables statistically evaluated were: fermentation kinetics and the level of volatile compounds produced. anova tests were carried out with the Statgraphics (Manugistics Inc., Rockville, MD, USA) software.

Results

Effect of IASF and HASF conditions without nitrogen addition on production of volatile compounds

The production of volatile compounds at HASF was statistically different compared with volatile compounds produced in industrial *A. tequilana* sugar fermentation (IASF). The production of ethyl acetate and amyl alcohols was lower in IASF (Fig. 1), while there were no statistically significant differences between acetaldehyde, propanol and amyl alcohol production at HASF and IASF (Fig. 1). Methanol was lower in IASF than in HASF, but it was not produced during fermentation (Fig. 1). Thus, the concentration observed at the beginning came from

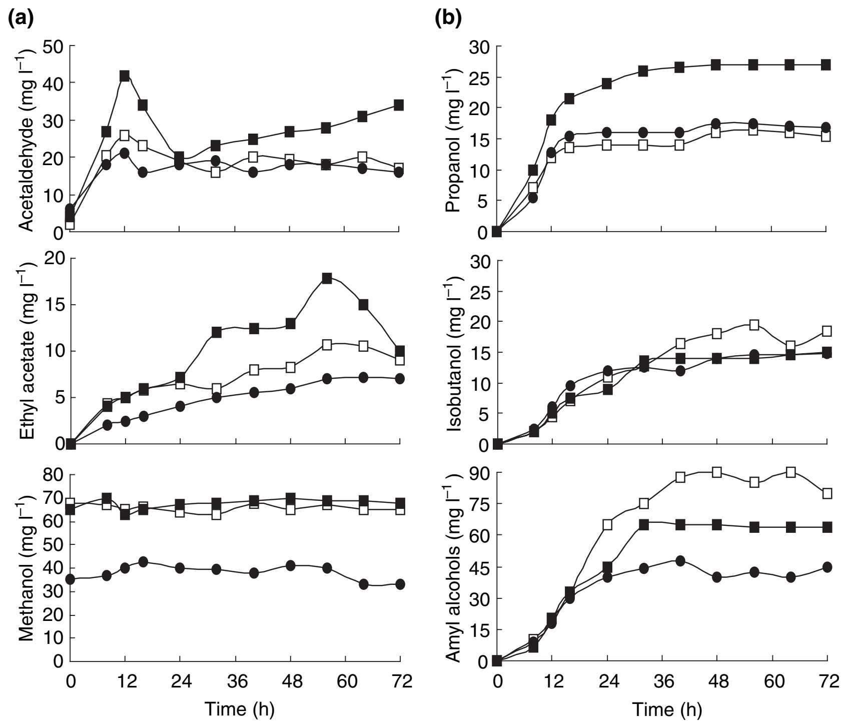

Figure 1 Volatile compound production in fermentations at high *Agave tequilana* sugar concentration (170 g l^{-1}) at 35°C, with (■) and without (□) nitrogen addition (ammonium sulfate (323·4 mg l^{-1}) and amino acids (113·72 mg l^{-1}), and fermentation at industrial *Agave tequilana* sugar concentration (80 g l^{-1}) at 35°C (●).

the agave raw material, or possibly it was produced by the break down of methoxyl groups during the cooking step.

Effect of nitrogen addition with a mixture of ammonium sulfate and amino acids at HASF conditions on production of volatile compounds

When nitrogen was added in the exponential phase of growth at HASF with a mixture of ammonium sulfate and amino acids, there was an increase in the production of acetaldehyde, ethyl acetate and propanol, and there were statistically significant differences in the concentration of these compounds at HASF with and without nitrogen addition.

In case of acetaldehyde, it reached a peak (40 mg l^{-1}) at 12 h of fermentation, followed by a decrease in production until 24 h, then finally an accumulation was observed (Fig. 1). In addition, the production of ethyl acetate increased when nitrogen addition was added (Fig. 1). With regard to the production of higher alcohols, when nitrogen was added, the production of propanol increased while isobutanol and amyl alcohols production decreased (Fig. 1). There were no statistically significant differences between the methanol concentrations with and without nitrogen addition.

Effect of different nitrogen sources added at HASF conditions on fermentation kinetics

Due to the differences observed with nitrogen addition at HASF, and because of the nature of the nitrogen source added, which consist of a mixture of ammonium sulfate and amino acids (inorganic and organic nitrogen source), an experiment was performed in order to evaluate the effect of each specific nitrogen source: organic (glutamic acid) and inorganic (ammonium sulfate) at HASF.

In general, sugar consumption was faster with nitrogen addition than without (Fig. 2). The highest maximum specific sugar consumption rate was obtained with the addition of ammonium sulfate (6·55 g g^{-1} h^{-1}, Table 1), while the addition of glutamic acid and the mixture of ammonium sulfate and amino acids had maximum specific sugar consumption rates of 5·66 and 4·6 g g^{-1} h^{-1}, respectively (Table 1), and there were no statistically significant differences between these two last additions. A slower sugar consumption rate was observed without nitrogen addition (Fig. 2), where the maximum specific sugar consumption rate was 3·8 g g^{-1} h^{-1} (Table 1) and was statistically different to all the values of sugar consumption rates with addition of the different nitrogen sources.

It was generally observed that more ethanol was produced with nitrogen source addition than without.

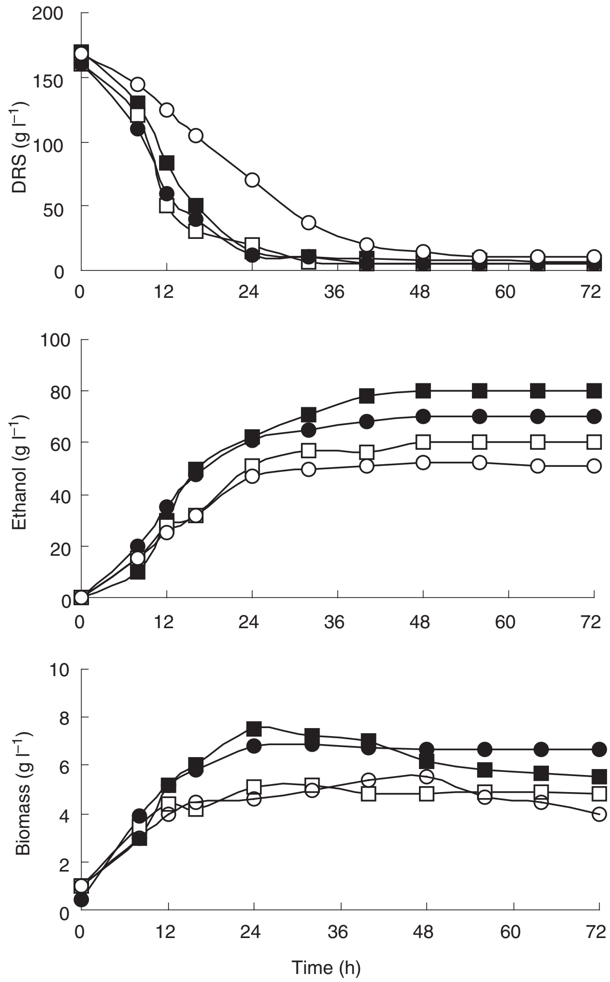

Figure 2 Evolution of sugar consumption as direct reducing sugars (DRS), ethanol, biomass production at high *Agave tequilana* sugar concentration (170 g l^{-1}) at 35°C, without (○) and with nitrogen addition at exponential phase of growth; glutamic acid (□), ammonium sulfate (●) and ammonium sulfate + amino acids (■).

According to Fig. 2 and Table 1, the addition of ammonium sulfate and amino acids mixture was the most effective for ethanol production and alcohol efficiency, followed by ammonium sulfate addition, and then less efficient for glutamic acid addition. The lowest ethanol production and efficiency was obtained without nitrogen (Fig. 2, Table 1). Inversely related to the behaviour of ethanol production, a higher maximum specific ethanol production rate was observed without nitrogen addition (1·5 g g^{-1} h^{-1}), followed by lesser rates when addition was performed with ammonium sulfate and glutamic acid (1·44 and 1·32 g g^{-1} h^{-1} respectively), which have no statistically significant differences in the ethanol production rate values of these two nitrogen sources. Finally the lowest maximum ethanol production rate was observed with

Table 1 Comparison of kinetic parameters of fermentations at high agave sugar concentration with and without nitrogen addition in the exponential phase of growth with respect to industrial agave sugar concentration

		HASF† – source of nitrogen added in the exponential phase of growth			
Kinetic parameter	IASF*	$(NH_4)_2SO_4$ + mixture of amino acids	Glutamic acid	$(NH_4)_2SO_4$	Without nitrogen addition
μ_{max} (h^{-1})*	0·36	0·45	0·36	0·42	0·34
q_{smax} ($g\ g^{-1}\ h^{-1}$)†	3·40	4·60	5·66	6·55	3·80
q_{pmax} ($g\ g^{-1}\ h^{-1}$)‡	1·70	1·20	1·32	1·44	1·50
Alcoholic efficiency (%)	80·0	94·5	84·0	89·0	74·0

IASF, industrial agave sugar fermentation; HASF, high agave sugar fermentation.
*Maximum specific growth rate.
†Maximum specific sugar consumption rate.
‡Maximum specific ethanol production rate.

the addition of ammonium sulfate and amino acids ($1{\cdot}20\ g\ g^{-1}\ h^{-1}$), which was statistically different to the value obtained without nitrogen addition and to the additions with ammonium sulfate and glutamic.

Biomass production was similar when the additions were carried out with ammonium sulfate and ammonium sulfate and amino acids, with maximum specific growth rates of 0·42 and 0·45 h^{-1}, respectively (Table 1), and there were no statistically significant differences. The addition of glutamic acid did not affect biomass production (Fig. 2), the maximum specific growth rates were also similar between glutamic acid addition and without nitrogen addition (0·36 and 0·34 h^{-1}, respectively, Table 1), and there were also no statistically significant differences between these two growth rates.

Effect of different nitrogen sources added at HASF conditions on production of volatile compounds

Regarding volatile compound production, in the fermentations at high *A. tequilana* sugar concentration with various nitrogen sources additions, the levels of acetaldehyde, ethyl acetate, propanol, isobutanol and amyl alcohols were all dependent upon the nitrogen source added.

In the case of acetaldehyde, there was a peak in production and an accumulation with each of the different nitrogen sources added. There was also an increase in propanol production and a decrease in isobutanol and amyl alcohols. As a result, Fig. 3, only the peaks of acetaldehyde production and the maximum acetaldehyde accumulated at 72 h of fermentation were presented. In the case of ethyl acetate, propanol, isobutanol and amyl alcohols, only the maximum production level was represented in Fig. 3.

It can be seen that the highest acetaldehyde peak production was observed with the addition of ammonium sulfate and amino acids (42·5 mg l^{-1}, Fig. 3). Then the acetaldehyde peak production was 41 and 28·5 mg l^{-1} for additions with ammonium sulfate and glutamic acid, respectively (Fig. 3), whereas without addition a smaller peak of 26 mg l^{-1} was observed (Fig. 3). There were no statistically significant differences between the acetaldehyde peaks with addition of ammonium sulfate and ammonium sulfate and amino acids, and between the

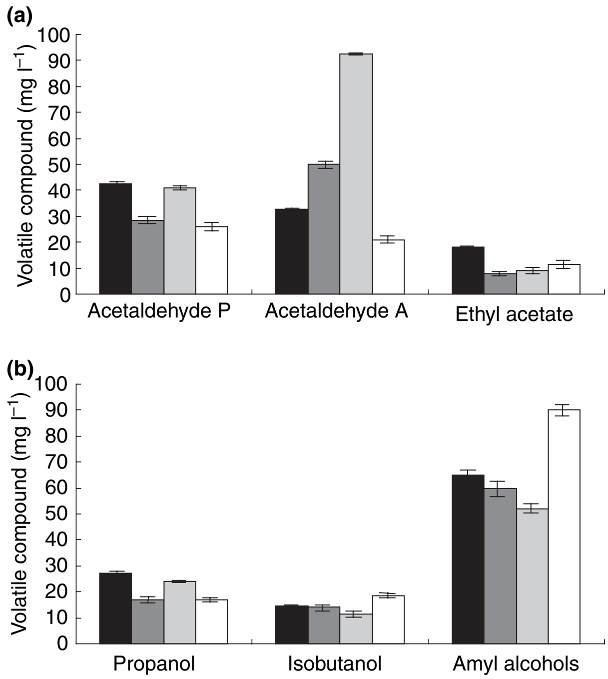

Figure 3 Production of volatile compounds in fermentations at high *Agave tequilana* sugar concentration at 35°C, without nitrogen addition (□), and with nitrogen addition at exponential phase of growth; glutamic acid (■), ammonium sulfate (■) and ammonium sulfate + amino acids (■). (a) Acetaldehyde peak (P) production, acetaldehyde accumulated (A) at the end of fermentation and maximum ethyl acetate produced. (b) Propanol, isobutanol and amyl alcohol production at the end of fermentation.

peaks with glutamic acid and without nitrogen addition. The concentration of acetaldehyde accumulated at 72 h of fermentation was highest with ammonium sulfate addition (92·5 mg l^{-1}, Fig. 3), followed by glutamic acid at 50 mg l^{-1}, the mixture of ammonium sulfate and amino acids at 32·5 mg l^{-1}, and finally 21 mg l^{-1} resulted when no nitrogen was added (Fig. 3), all the values of acetaldehyde accumulated were statistically different.

The highest ethyl acetate production was observed when a mixture of ammonium sulfate and amino acids was added (18 mg l^{-1}), followed by the fermentation without nitrogen addition (11·4 mg l^{-1}), whereas the fermentation with glutamic acid and ammonium sulfate addition produced lesser and similar amounts (8 and 9 mg l^{-1}, respectively). Only the ethyl acetate concentration obtained with addition of ammonium sulfate and amino acids was statistically different. With regard to higher alcohols, in the case of propanol and with the exception of glutamic acid addition, an increase was observed when nitrogen was added. The propanol production was 27, 24 and 17 mg l^{-1} for the mixture of ammonium sulfate and amino acids, ammonium sulfate and glutamic acid addition respectively (Fig. 3). The propanol production obtained without nitrogen addition was 17 mg l^{-1} (Fig. 3). There were no statistically significant differences in the propanol concentration between glutamic acid addition and without nitrogen addition. Conversely to that observed in propanol production, a decrease was observed in the production of isobutanol and amyl alcohols when nitrogen was added (Fig. 3). In the case of isobutanol production, the maximum levels observed were 14·5, 14, and 11·5 mg l^{-1} for the additions of the ammonium sulfate and amino acids mixture, glutamic acid, and ammonium sulfate, respectively (Fig. 3) and the isobutanol concentrations with additions of ammonium sulfate, glutamic acid and ammonium sulfate and amino acids were statistically similar. While without nitrogen addition the concentration obtained was 18·5 mg l^{-1} (Fig. 3) and this concentration was statistically different to the isobutanol concentrations obtained with the addition of the three different nitrogen sources. For amyl alcohol production, the levels were 65, 60 and 52 mg l^{-1} for the additions of ammonium sulfate and amino acids mixture, glutamic acid, and ammonium sulfate, respectively (Fig. 3). The amyl alcohol concentrations with additions of ammonium sulfate and ammonium sulfate and amino acids were statistically similar, while the amyl alcohol concentration with glutamic acid addition was statistically different. The highest concentration of amyl alcohols was obtained without nitrogen addition (90 mg l^{-1}, Fig. 3) and was statistically different to the concentrations obtained with the addition of the different nitrogen sources.

Comparison of volatile compounds with respect to the tequila standard

According to the Mexican Ministry of Commerce and Industry (Regulations: NOM-006-SCFI-2005, 2006), the concentrations of the regulated volatile compounds in tequila are to be reported as milligrams of volatile compound per 100 ml of anhydrous alcohol produced. The ratio of the volatile compounds per 100 ml of anhydrous alcohol was calculated with both the final ethanol and volatile compound concentrations that were reached in all the fermentations. These results are shown in Table 2. It can be seen that in general the concentrations of ethyl acetate, methanol and the higher alcohols were higher in fermentations at IASF and HASF than with addition of nitrogen (Table 2). Conversely, the concentration of acetaldehyde was higher in fermentations with the addition of nitrogen than without (Table 2). In review of each of

Table 2 Comparison of the level of regulated compounds produced in IASF and HASF tequila fermentations

					Source of nitrogen added		
Volatile compound	Units	Tequila standard	IASF	HASF	$(NH_4)_2SO_4$ + mixture of amino acids	$(NH_4)_2SO_4$	Glutamic acid
Ethanol	% Volume	35-55	5·06	6·50	10·12	8·80	7·60
Acetaldehyde	mg 100 ml^{-1} a.e.	0-40	35·5	32·5	32·0	104·4	66·0
Ethyl acetate	mg 100 ml^{-1} a.e.	2-270	14·0	17·6	17·7	10·1	10·5
Methanol	mg 100 ml^{-1} a.e.	30-300	79·0	103·8	64·0	74·5	85·6
n-Propanol	mg 100 ml^{-1} a.e.	–	35·5	26·0	26·6	27·0	22·4
Isobutanol	mg 100 ml^{-1} a.e.	–	29·6	28·6	14·3	13·0	18·4
Amyl alcohols	mg 100 ml^{-1} a.e.	–	89·0	139·4	64·2	58·7	79·0
Higher alcohols*	mg 100 ml^{-1}a.e.	20-400	154·1	194·0	105·1	98·7	119·8

IASF, industrial agave sugar fermentation; HASF, high agave sugar fermentation; a.e., anhydrous ethanol.

*Higher alcohols: propanol + isobutanol + amyl alcohols.

cases, it is only the concentration of acetaldehyde, especially in the fermentations at high sugar concentration with the additions of glutamic acid and ammonium sulfate that was higher than the level allowed by the tequila norm (Table 2).

Discussion

In previous studies, the addition of nitrogen in fermentations at high sugar concentration has been performed with different type of sugars. The main conclusion of these studies was that if an adequate source of nitrogen was added, the fermentation length can be reduced by increasing sugar transport (Salmon *et al.* 1993), causing an accelerated fermentation rate, alcohol and some volatile compounds production (Bely *et al.* 1990; Thomas and Ingledew 1990; Jiranek *et al.* 1995; Buescher *et al.* 2001; Arrizon and Gschaedler 2002; Mendes-Ferreira *et al.* 2004; Beltran *et al.* 2005). Recently, a high correlation between sugar and assimilable nitrogen consumption rate was found (Malacrino *et al.* 2005). Nevertheless, in case of tequila, no studies have been performed to date regarding volatile compound production and fermentation kinetics with the addition of nitrogen of different nature, in the exponential phase of the growth in fermentations at high *A. tequilana* sugar concentration (HASF).

Comparing the volatile compounds produced at IASF and HASF conditions (without nitrogen addition), with an equal initial nitrogen concentration in both fermentations, the synthesis of isobutanol and amyl alcohols was increased as the C/N ratio increased. This behaviour has been observed in similar studies, where in wines an increase in the synthesis of higher alcohols has been observed when the exhaustion of high residual total assimilable nitrogen occurred (Malacrino *et al.* 2005). Furthermore, an increase in higher alcohols has also been observed when the level of yeast assimilable nitrogen was low in synthetic must in fermentations with high sugar concentrations (Wang *et al.* 2003). This behaviour was also observed in tequila fermentation in the work of Pinal *et al.* (1997). Conversely, our study found that propanol synthesis at IASF and HASF conditions was inhibited by the high sugar concentration and as a consequence similar levels were produced in both sugar concentrations. For acetaldehyde synthesis, similar levels were produced in IASF and HASF fermentations, thus an inhibition of fermentation was also observed. The phenomenon of inhibition of fermentation by high sugar concentration is well documented by the works of Bisson (1999), and Bisson and Butzke (2000).

The additions of nitrogen from different sources at HASF modified the synthesis behaviour of all the compounds except methanol. Regarding the decrease of the isobutanol and amyl alcohol synthesis caused by nitrogen addition, a similar result has been found in the work of Beltran *et al.* (2005), where a mixture of ammonium and amino acids was added at high sugar concentrations (200 g l^{-1}) during the early fermentation step. Higher alcohols can be produced by catabolic conversion of the branched chain amino acids (via Ehrlich) or by the anabolic formation of these amino acids *de novo* from sugars (Äyräpää 1971). Thus, the increase in synthesis of higher alcohols is a result of low assimilable nitrogen as more carboxylic acids (α-cetoacids) are available for the production of higher alcohols than for amino acids synthesis (Walker 1999). In our case, with and without nitrogen addition, it is possible that the synthesis of amyl alcohols and isobutanol occurred by anabolic formation of amino acids. Converse to isobutanol and amyl alcohol production, propanol synthesis increased with the addition of the mixture of ammonium sulfate and amino acids, and ammonium sulfate alone. In the work of Beltran *et al.* (2005), an increase in propanol synthesis was also observed with the addition of ammonium and amino acids. However, the addition of glutamic acid did not stimulated propanol synthesis. It has been found that ammonium sulfate is better assimilated than glutamic acid, tryptophan and other amino acids (Beltran *et al.* 2005). Additionally, it has been found that nitrogen requirements varied between the yeast strains (Jiranek *et al.* 1995; Manginot *et al.* 1998), which was also reflected in differences in volatile compound production in wine (Romano *et al.* 2003; Malacrino *et al.* 2005) and synthetic grape must (Wang *et al.* 2003). This could explain why glutamic acid addition only affects amyl alcohols and isobutanol. Furthermore, it is possible that the relationship between higher alcohols and C/N ratio was different for propanol production with respect to isobutanol and amyl alcohols.

With regard to acetaldehyde production, an increase in concentration was observed with the addition of different nitrogen sources at HASF. A similar trend was observed when a mixture of ammonium and amino acids was added to synthetic grape must at high sugar concentration in the early step of fermentation (Beltran *et al.* 2005). It has been found in previous work that, in tequila fermentation at higher agave sugar concentration, the addition of nitrogen increased sugar consumption and fermentation rate (Arrizon and Gschaedler 2002). As a result more pyruvate was produced because of a rapid transformation of sugars by glycolysis to pyruvate, which then was transformed to acetaldehyde (Pronk *et al.* 1996). This could explain the accelerated acetaldehyde production in each of the nitrogen additions in our study. It was also observed that the consumption of sugar finished at 24 h, reaching the lowest sugar concentration (Arrizon and Gschaedler 2002). As the enzyme alcohol dehydrogenase consumes acetaldehyde and decreases its activity when sugar concentration is low (Reid and Fewson 1994), it could be possible that after 24 h of fermentation, alcohol dehydrogenase has low activity and for that reason acetaldehyde started to accumulate.

The synthesis of ethyl acetate at HASF and IASF fermentations was increased as more carbon source was available. Furthermore an increase in ethyl acetate was observed with the addition of some nitrogen sources. In the work of Fujiwara *et al.* (1998) it was found that ester synthesis increased as available amino nitrogen increases. Furthermore with nitrogen addition in the early step of fermentation using a mixture of ammonium and amino acids in synthetic grape must, an increase in ester synthesis was also observed (Beltran *et al.* 2005). However, in our study the relationship between ethyl acetate synthesis and the source of nitrogen added was unclear, possibly because only ethyl ester was measured, but not the other esters obtained by the synthesis from higher alcohols. It has been found in distilled Marula beverage that when the temperature changed from 15 to 30°C, ethyl acetate synthesis was decreased (Fundira *et al.* 2002). As the fermentations at HASF conditions were carried out at 35°C, it is possible that temperature has an effect as well.

With regard to the effect of nitrogen sources addition on the rate of sugar consumption, biomass and ethanol production, it has been found that the *Saccharomyces* yeast had preference for ammonium ions than amino acids (Walker 1999; Beltran *et al.* 2005). Additionally, it has been observed that growth and fermentation rates improved as yeast assimilable nitrogen was increased (Wang *et al.* 2003). Also that ammonium salt addition at high sugar fermentation was more effective for sugar transport activation because of the induction of sugar transport proteins biosynthesis (Salmon 1989; Mauricio and Salmon 1992; Salmon *et al.* 1993) and the reduction of the length of fermentation (Mendes-Ferreira *et al.* 2004; Beltran *et al.* 2005). Additionally, it has been observed that ammonium was the preferred nitrogen source and that as glutamine and tryptophan were better assimilated than other amino acids (Beltran *et al.* 2005). This could explain why the addition of the mixture of ammonium sulfate and amino acids, and ammonium sulfate alone were more effective than glutamic acid on sugar consumption, as well as ethanol and biomass production, which was reflected also in their respective kinetic parameters, with the exception of the maximum specific rate of ethanol production. As the maximum specific rates were calculated in the relationship to the biomass generated, the values depended on biomass concentration. In our case, the maximum specific ethanol production rate depended more on biomass concentration than the maximum specific sugar consumption rate.

When the addition of nitrogen was performed with ammonium sulfate and glutamic acid the concentration of acetaldehyde was higher than the level allowed by the tequila norm (NOM-006-SCFI-2005, 2006). Nevertheless, the problem of the elevated acetaldehyde could be solved by an efficient method of distillation (Prado-Ramírez *et al.* 2005). Therefore, the addition of nitrogen performed in the conditions studied could be used to produce tequila with the regulated compounds in the range allowed. However, the changes observed in the synthesis of volatile compounds caused by nitrogen addition, can modify the sensorial quality of the final product, as has been observed with low and high levels of acetaldehyde and higher alcohols (Miyake and Shibamoto 1993; Lambrechts and Pretorious 2000). Therefore, additional research will be performed to study this effect.

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