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The Effect of SO_2 on the Production of Ethanol, Acetaldehyde, Organic Acids, and Flavor Volatiles during Industrial Cider Fermentation

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SO_2 is widely used in cider fermentation but also in other alcoholic beverages such as wine. Although the authorized limit is 200 ppm total SO_2, the International Organizations recommend its total elimination or at least reduction due to health concerns. Addition of SO_2 to apple juice at levels frequently used in industrial cidermaking (100 mg/L) induced significantly higher acetaldehyde production by yeast than that obtained without SO_2. Although the practical implications of acetaldehyde evolution under cidermaking conditions has been overcome by research and few data are available, this compound reached levels in two 2000 L bioreactors that may have prevented the occurrence of simultaneous alcoholic and malolactic fermentation. It was observed that malolactic fermentation had a positive effect promoting reduction of acetaldehyde levels in cider fermented with juice, SO_2-treated or not. The addition of SO_2 clearly delayed malolactic fermentation comparing to the control, affecting not the onset of the malolactic fermentation but the rate of malic acid degradation. This compound, however, had a stimulatory effect on alcoholic fermentation.

KEYWORDS: Cider; SO_2; industrial fermentation; malolactic

INTRODUCTION

Sulfur dioxide (SO_2) is used in cidermaking as an antioxidant, an inhibitor of oxidizing enzymes. It combines with products of previous oxidation, prevents darkening and some hazes, as well as acting as an inhibitor of a wide range of microorganisms. Research has demonstrated the presence of sulfur dioxide-binding compounds derived from three sources: (i) fruit components (such as glucose and arabinose), (ii) metabolites produced by bacteria (such as *Gluconobacter* and *Acetobacter*, growing in rotting fruit), (iii) during fermentation, *Saccharomyces* spp. can produce acetaldehyde, and pyruvic and 2-oxoglutaric acids (*1*).

The total SO_2 added to cider consists of various forms of this compound (bound and free forms) in equilibrium, with different antimicrobial activity; the degree and speed of this equilibrium being dependent on pH and temperature. At pH 3−4, free SO_2 consists mainly of the bisulfite anion (HSO_3^{-1}), a small fraction of molecular SO_2 (H_2SO_3), which is considered the active form of SO_2, and a negligible amount of sulfite anion (SO_3^{-2}). Carbonyl compounds (mainly acetaldehyde, pyruvic acid and 2-oxoglutaric acid) bind with free SO_2, (especially the bisulfite ion) to form complex compounds (bound SO_2). Bound SO_2 shows a weak antimicrobial function. When the fact that acetaldehyde has a strong affinity for SO_2 is taken into account, the product bisulfite-acetaldehyde resulting from the addition represents the majority of the total SO_2. The binding of the bisulfite ion and acetaldehyde reduces the amount of free SO_2 available, but this bound form may also be inhibitory to lactic acid bacteria conducting malolactic fermentation, probably due to SO_2 release coupled to bacterial metabolism of the acetaldehyde moiety (*2*). Microorganisms vary greatly in their sensitivity to SO_2: Bacteria, particularly Gram-negative rods, are markedly sensitive; aerobic microorganisms are more sensitive than fermenting microorganisms (*1*).

As reviewed (*3*) for cider, it seems essential that only a minimum effective quantity of SO_2 should be used. The amount required depends on the pH of the apple juice and the concentrations of the sulfite-binding compounds present, within the legally permitted limit of 200 ppm of total sulfur dioxide (European Commission, U. S. Food and Drug Administration). As practical recommendations (*3*), the concentrations required would be: pH 3.0−3.3, 75 ppm; pH 3.3−3.5, 100 ppm; pH 3.5−3.8, 150 ppm. The juice should be left to equilibrate for at least 6 h, and the free SO_2 should be determined. From toxicity tests on cider yeasts, it was shown that for effective sulfiting, a minimum residual free SO_2 content of 30 ppm at pH 3.5 was necessary (*1*). Low levels of free SO_2 indicate the presence of excessive quantities of sulfite-binding compounds, due to the use of concentrated juice (formed during heat treatment) (*4*) or the use of rotten fruit, in which acetic acid bacteria, mainly *Acetomonas* spp., produce oxo-acids, which bind strongly with sulfur dioxide. Excessive quantities of free SO_2 will cause delays in the onset of fermentation.

The use of SO_2 in cidermaking has been considered absolutely necessary. Nevertheless, International Organizations have ex-

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tended their recommendations to its total elimination or at least reduction due to health concerns (JEFCA: Joint FAO/WHO Expert Committee on Food Additives). To this aim, alternative methods such as apple juice storage under N_2 atmosphere (to avoid O_2 contact, which acts as a source of oxidations and microbial spoilage), the use of ascorbic acid, and yeast inoculation have become useful tools to achieve a better microbial stability.

In this work, cider fermentation at an industrial scale (2000 L, using juice diluted from concentrate and a yeast dried inoculation) was performed with (100 mg/L) and without SO_2, with the goal of verifying the practical implications and the role of this compound under industrial cidermaking conditions.

MATERIALS AND METHODS

Fermentation Conditions. Fermentations were carried out in two stainless steel bioreactors (2000 L capacity). In one bioreactor, juice was treated with SO_2 (100 mg/L), while the other was maintained in the same conditions but in the absence of this compound. The bioreactors were held in the cellar of the commercial cider maker Escanciador, S. A. (Villaviciosa, Principado de Asturias, Spain), at room temperature (approximately 15 °C).

Concentrated apple juice (1360 g/L, bright, enzymatically treated) was diluted to 1080 g/L (final density), pH 3.7. Juice treated with SO_2 (100 mg/L) was left at room temperature 24 h before inoculation (free SO_2 content was then 30 mg/L, see below). An initial volume of fermentation (100 L) was prepared (without SO_2), by adding a commercial active-dry yeast strain (500 g) of *Saccharomyces cerevisiae* subsp. *bayanus* (strain Pasteur Institute, Paris, 1969, "Champagne", supplied by Novo Ferment, Switzerland), following the manufacturer's recommendations. Then, 50 L of this volume were dispensed to each bioreactor, and juice (SO_2 treated or not) was added to start fermentation. After 40 days, the fermentation media were moved to other bioreactors and held in the same conditions.

Sample Preparation and Analytical Methods. Free SO_2 content in juice was determined by iodometry. Samples were collected periodically from the sampler at the bottom of each tank, filtered immediately through a 0.45-μm membrane and frozen (−20 °C) in 2 mL vial replicates until analysis. Samples were analyzed in duplicates, with coefficients of variation less than 6%. Organic acids in samples were determined by HPLC (Waters, Alliance 2690) equipped with a photodiode array detector (Waters 996), as previously described (*5*, *6*). Volatile compounds with boiling points lower than 145 °C were analyzed using a gas chromatograph (GC-14B, Shimadzu) equipped with an FID detector and an auto injector (AOC-20i, Shimadzu), fitted with a Supelcowax 10 (Supelco) column (60 m × 0.25 mm i.d., phase thickness 0.25 μm), as previously described (*7*). Butyl acetate was used as internal standard.

RESULTS AND DISCUSSION

Effect on Alcoholic Fermentation. As indicated, the antimicrobial effect of SO_2 is well known, playing a selective role on microbiota during fermentation, with fermentative yeasts more resistant than not fermentative yeasts and bacteria. The precise nature of the inhibitory action of SO_2 on microorganisms is not, however, completely understood (*1*), although SO_2 reacts directly with thiamine, reducing the available level of this vitamin to microorganisms (*8*).

Under our working conditions (reconstituted concentrate apple juice and yeast inoculation), the concentration of SO_2 used did not cause a delay on the onset of the alcoholic fermentation. Indeed, a slightly stimulatory effect could be observed in the first 10 days (**Figure 1**). Alcoholic fermentation in both cases was completed in 14 days. It has been suggested that the reason SO_2 can stimulate fermentation by *Saccharomyces* in wine lies in the inhibition of the competing polyphenol oxidase. This is

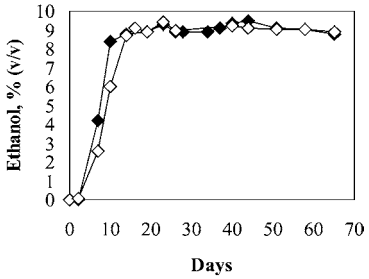

Figure 1. Evolution of alcoholic fermentation. Solid symbols, with SO_2; hollow symbols, without SO_2.

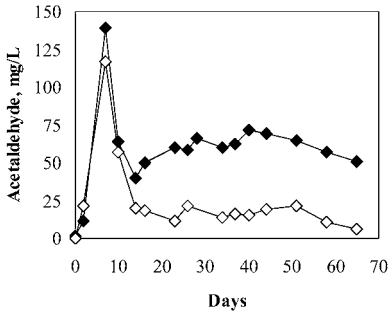

Figure 2. Acetaldehyde production in juice, SO_2-treated (◆) and not treated (◇).

a grape must enzyme that competes directly for available dissolved oxygen, therefore making oxygen more readily available for *Saccharomyces*, and not in the inhibition of wild (non *Saccharomyces*) yeast and bacteria (*9*). In addition, an excess of free carbonyl compounds causes inhibition of yeast fermentation (*8*), neutralized by SO_2 addition.

Effect on Acetaldehyde Formation. Acetaldehyde evolution during both fermentation processes is shown in **Figure 2**. As SO_2, acetaldehyde in cider exists in free and bound forms; it should be borne in mind that, due to the analytical method employed (gas chromatography), the values presented here corresponded to free acetaldehyde. Acetaldehyde is one of the most important sensory carbonyl compounds in alcoholic beverages and is formed during alcoholic fermentation by yeast. When present in excess, acetaldehyde imparts an undesirable green, grassy, applelike aroma, which is usually masked by the addition of SO_2. The flavor threshold of acetaldehyde in cider has been established as approximately 30 mg/L (*10*). Differences in acetaldehyde production depend on the yeast species or strain used, but factors such as temperature, oxygen, and SO_2 concentrations affect its production by yeast as well (*2*). Sugar is the primary substrate, but metabolism of amino acids such as alanine also contributes to the formation of this compound. In addition, it is also formed from the oxidation of ethanol by film yeasts. Acetaldehyde is excreted mainly during the growth period and can be recatabolized, although the yeast is not able to metabolize the acetaldehyde bound to SO_2 (*2*, *4*).

As expected (**Figure 2**), maximal values were reached seven days after the onset of fermentation, and then this compound was re-adsorbed. On the basis of the results obtained, it could be confirmed that the addition of SO_2 induces acetaldehyde formation by yeast, as previously reported in winemaking (*11*, *2*). It was previously suggested that this SO_2-induced production of acetaldehyde may be related to SO_2 resistance in yeasts (*2*). It is noteworthy that the use of SO_2 under industrial cidermaking conditions, applied at levels usually employed in cider factories, and not exceeding published recommendations, rendered significantly higher acetaldehyde concentrations than the control

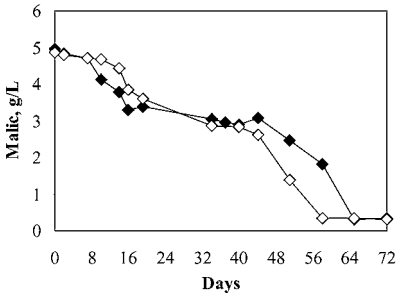

Figure 3. Malic acid degradation in the sulfited (solid symbols) and not sulfited juice (hollow symbols).

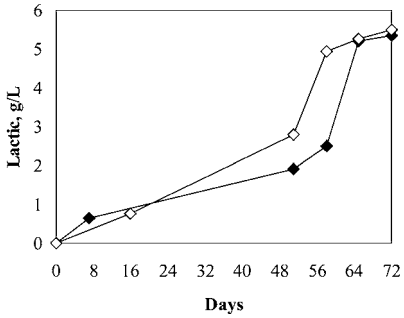

Figure 4. Lactic acid formation in juice with (◆) and without SO_2 (◇).

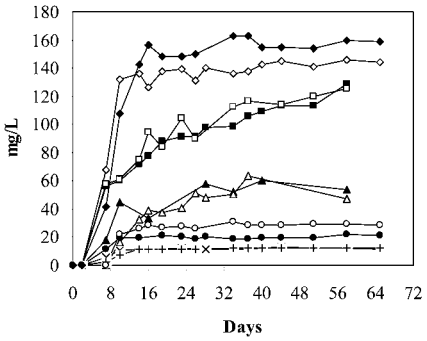

Figure 5. Volatile compounds' evolution during fermentation: (◆ ◇) 2- and 3-methylbutyl alcohols; (■ □) methanol; (▲ △) ethyl acetate; (● ○) 1-propanol. Solid symbols, with SO_2; hollow symbols, without SO_2. (+) 2-methyl-1-propanol in not sulfited juice and (×) 2-methyl-1-propanol in sulfited juice.

without SO_2. Although cidermaking technologists tend to use SO_2 to mask excessive levels of this compound, which negatively affect the organoleptic properties of cider, the addition of SO_2 (100 mg/L) to the juice induces acetaldehyde formation in industrial cider production. The highest amount measured corresponded to 139 mg/L in the presence of SO_2, while 116.9 mg/L was reached as maximum in the absence of this compound. Furthermore, when fermentation was carried out without SO_2, and once the compound was re-adsorbed (i.e., from day 14, corresponding to the end of alcoholic fermentation), it maintained almost constant levels near 20 mg/L, until the last 20 days of fermentation, when the concentration decreased to 6 mg/L. With SO_2, higher levels were measured throughout the fermentation process. After day 14, a second increment could be observed (from 39.9 to 71.4 mg/L), then decreasing again from day 40 until the end of fermentation, with a final value of 50.6 mg/L. These variations could be related to changes taking place during malolactic fermentation. A reduction in acetaldehyde concentration from 17 mg/L initially to 1.5 mg/L at the end of the malolactic fermentation in wine has been reported (*12*). It was also demonstrated that some wine lactic acid bacteria can catabolize SO_2-bound and/or free acetaldehyde (*13*). As shown in **Figure 3**, the occurrence of complete malolactic fermentation may be related to the reduction of the acetaldehyde final concentration in both bioreactors. The levels of free acetaldehyde, as well as other carbonyl compounds that bind SO_2, will be critical to achieving a correct second sulfiting step in cider production, once the fermentation process is completed. The practical implications of acetaldehyde evolution under cidermaking conditions has been overcome by research, and few data are available. To date, there is a lack of available studies that have focused on cider fermentation under industrial conditions. Nevertheless, acetaldehyde content has also been related to stuck and slugglish fermentations (*8*), because it is toxic even at low levels and may have a synergistic effect with other factors.

Effect on Malolactic Fermentation. As mentioned, complete malolactic fermentations took place in both bioreactors (**Figure 3**). However, the addition of SO_2 clearly delayed this process by 8 days (day 58 vs. day 66) when compared to the control. Lactic acid evolution (**Figure 4**) showed the same pattern. As indicated, the effect of SO_2 on malolactic fermentation has been related to its inhibitory effect over the wild population of lactic acid bacteria initially present and to the effect of SO_2-bound acetaldehyde on lactic acid bacteria. In this case, the addition of SO_2 does not seem to affect the time of the onset of the malolactic fermentation, but mainly the duration of the process. It should be noted that *S. cerevisiae* may also metabolize malic acid (*14*, *6*). The concentration of acetaldehyde is critical for the malolactic fermentation, as it has been demonstrated that high levels (>100 mg/L) may inhibit growth of heterofermentative lactic acid bacteria, while low levels (<100 mg/L) stimulate growth of these bacteria (*2*). Thus, the acetaldehyde levels reached in both bioreactors during the first days of the cidermaking process may have prevented the development of simultaneous alcoholic and malolactic fermentations. It is important to underline that, in both cases, malic acid degradation stopped for 25 days and restarted at the same time in both bioreactors but with a slower rate when the juice was SO_2-treated. This result again supports the need for the development of a proper starter culture for malolactic fermentation, suitable to be used in industrial cider fermentation processes (*15*).

Effect on Other Volatile Compounds. The compounds analyzed in this work are mainly produced during yeast metabolism, having an important influence on the organoleptic characteristics of cider. Among the volatile compounds tested (**Figure 5**), few differences could be found between industrial scale cider fermented with and without SO_2. Methanol, ethyl acetate, and 2-methyl-1-propanol contents were not significantly influenced by the use of SO_2. On the contrary, 1-propanol and 2- and 3-methylbutyl alcohols were affected by SO_2 treatment. 1-Propanol may be formed from the carbon skeleton corresponding to the amino acid threonine by transamination, while 2- and 3-methyl-butanol are derived from isoleucine and leucine, respectively. In winemaking, higher amounts of 1-propanol were also reported in wines fermented without SO_2 (24.33 vs 12.81 mg/L) although in that case, 2- and 3-methyl-butanol were more abundant in wines fermented with SO_2 (*11*).

Effect on Other Organic Acids. Organic acid composition and concentration of each acid in cider plays an important role affecting the quality of the final product (*10*).

Pyruvic acid (an intermediate in the Embden−Meyerhof−Parnas pathway and precursor to many other substances) is another carbonyl compound that binds SO_2. This acid showed a different pattern dependent on the apple juice treatment. When SO_2 was present, this acid could be detected from day 16 (0.36

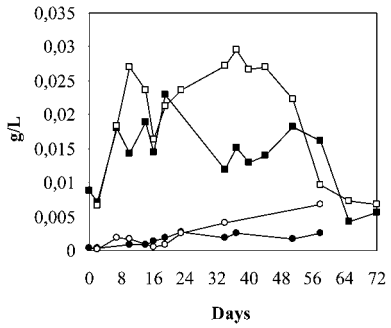

Figure 6. Shikimic (■ □) and fumaric (● ○) acids profiles with (solid symbols) and without (hollow symbols) SO_2.

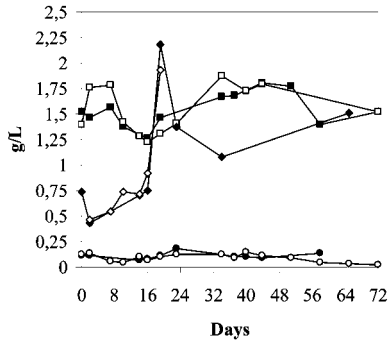

Figure 7. Quinic (■ □), succinic (◆ ◇), and citric (● ○) acids during fermentation in the sulfited (solid symbols) and not sulfited (hollow symbols) juice.

g/L), increasing until day 37 (0.43 g/L) and then decreasing until the end of malolactic fermentation (0.33 g/L final concentration). When fermentation was carried out in the absence of SO_2, detectable levels could not be measured during the fermentation processes. This lack of information suggests that addition of SO_2 apparently induces pyruvic acid production as well, taking into account that, in the nontreated juice, no detectable levels of this intermediate compound could be found. It has been previously noted that increased pyruvate excretion after SO_2 addition with the deficiency in thiamine is caused by SO_2 (*14*).

A slightly higher acetic acid content in the nontreated juice (0.7 g/L) was reached compared to the sulfited (0.6 g/L) juice. This may be related to the slower rate of the alcoholic fermentation observed in the absence of SO_2.

It was reported that the presence of 0.5 and 1.5 g/L of fumarate in wine at pH 3.0 inhibited malolactic fermentation, and that the bactericidal effect of this acid and SO_2 seemed to be additive (*16*). The bactericidal effect of fumaric acid added in cider has been recently investigated (*17*). In the present work, it was observed that fumaric acid formation during fermentation (**Figure 6**) was higher (0.008 g/L) in juice without SO_2 treatment than in the treated juice (0.002 g/L), but at significantly lower levels than those reported, because no more acid was added, and thus a similar impact on the malolactic fermentation could not be expected. Differences in shikimic acid profiles were observed in the middle stages of fermentation, but reached similar final concentrations (**Figure 6**). No remarkable differences could be assigned to SO_2 usage in the evolution of the other acids tested: quinic, succinic, and citric (**Figure 7**).

CONCLUSIONS

The results shown in this work were obtained at industrial fermentation scale, a scale at which very few studies have been previously undertaken due to the difficulty of making use of industrial equipment for experimental purposes. By use of reconstituted apple juice without SO_2 addition, fermentation rendered lower acetaldehyde content and shortened the duration of MLF compared to the SO_2-treated juice, under the same conditions. The widespread use of concentrate apple juice and yeast inoculation in industrial cider production, along with the application of higher hygienic measures in the processing plant, have led to a reduction in indigenous microbiota. This has facilitated changes in the application of traditional practices such as juice sulfiting. Reduction of acetaldehyde content by fermentation could be used as an alternative way to SO_2 addition to cider with the aim of masking the flavor of this compound. The microbial reduction is irreversible in contrast to variations in the concentration of this compound due to SO_2 loses by evaporation. The use of starter cultures for malolactic fermentation properly adapted to the specific features of cider would allow a better control over this important process.

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