# Effect of sulphur dioxide and ethanol on acetic acid bacteria occurring in wine technology

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**Summary**
This study aimed to analyse the sensitivity of selected acetic fermentation bacteria *Gluconobacter oxydans* (CCM 3618) and *Acetobacter aceti* (CCM 3620$^T$), which are found in wine technology, to ethanol and ammonium bisulfite at concentrations of 0–96 % vol. (ethanol) and 0–100 g·l$^{-1}$ (ammonium bisulfite). The ability to eliminate the bacteria was investigated by plating methods and by measuring growth curves in a complete medium. The elimination study using the plating method involved two different procedures. First, inhibitory substances were applied to sterile discs and the resulting inhibition zones (disc diffusion method) were measured, while the second procedure involved the application of different concentrations of inhibitory substances to the inoculated medium. The results showed the degree of resistance to the applied substances, with *A. aceti* being more resistant to both inhibitors than *G. oxydans*. Instead of suppressing growth, low concentrations (ethanol 0.8 % (v/v), ammonium bisulfite 0.8 g·l$^{-1}$) of the inhibitory agents had the opposite effect. These results are particularly important for the wine industry, where efforts are being made to eliminate undesirable acetic acid bacteria by the application of sulfur dioxide or ethanol.

**Keywords**
acetic acid bacteria; inhibition; ethanol; sulfur dioxide

Sulfur dioxide ($SO_2$) is widely used as a preservative in the food industry because of its antibacterial and antifungal properties [1]. Its mechanism of action is probably a combination of reduction of co-factors and vitamins, reduction of disulfide bridges in peptides and disruption of nucleic acids [2]. Sulfur dioxide has the strongest effect on bacteria, especially on many strains of lactic acid bacteria, while its effectiveness against acetic acid bacteria is somewhat lower. The mechanism of inhibition is as follows: First, molecular $SO_2$ encounters adenosine triphosphate (ATP) hydrolases, which are in the cytoplasmic membrane. This causes a rapid decrease in the concentration of ATP in the cytoplasm. Subsequently, $SO_2$ passes through the membrane into the cytoplasm as a neutral molecule by free diffusion or by active transport through ATPases [3]. Several binding sites for sulfites are present on the cell surface. After crossing the membrane to the cytoplasm, sulfites disrupt a number of essential molecules, enzymes and processes. The most important mechanisms include modification of proteins by disruption of disulfide bridges and induction of transcriptional mutations in nucleic acid. Divol et al. [4] described inhibition of the activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase, which is an essential component of glycolysis, as a key mechanism. Its inhibition causes a reduction in the production of ATP as well as the inhibition of regeneration of nicotinamide adenine dinucleotide hydride (NADH). However, this process has been found to be reversible. Other inhibited proteins include, for example, alcohol dehydrogenase, nicotinamide adenine dinucleotide ($NAD^+$)-glutamate dehydrogenase or some ATPases. Furthermore, sulfites in the cytoplasm damage co-enzymes and co-factors such as $NAD^+$,

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flavin adenine dinucleotide ($FAD^+$), thiamine or menadione. Due to the polyvalent effect of sulfur dioxide on microorganisms, completely resistant microbial strains have not yet been encountered. However, some strains show partially reduced susceptibility to $SO_2$ and associated resistance to other fungicides. Soon after the resistance in some strains was described, it was found that the resistance mechanisms were polygenically controlled and dominantly heritable, even in the absence of selection pressure. Described mechanisms of resistance included oxidation or a converse reduction of sulfites, increased production of acetaldehyde, which is characterized by a high affinity to $SO_2$, increased efflux pumping of $SO_2$ out of the cell or transition to a viable but non-culturable cell phase.

Ethanol affects microbial growth even at low concentrations. Electron microscopy observations showed intense morphological modifications of cells in the presence of ethanol. This clearly indicated that cell wall changes are partly responsible for the antibacterial effect of ethanol. High temperature and presence of ethanol are important limiting factors for acetic acid bacteria. These bacteria must tolerate a high initial ethanol concentration, which inhibits the growth of acetic acid bacterial cells [5]. At high temperatures, denaturation of essential enzymes and damage to cell membranes occur [6]. With these limiting factors, acetic acid bacteria cell growth is restricted and acetic acid yield is reduced. The use of ethanol at relatively high concentrations (60–70 % (v/v)) as a disinfectant is well known. However, few studies reported the effects of low concentrations of ethanol as an antimicrobial agent or a preservative in food products. Most of the published work on the efficacy of ethanol as a food preservative concerned its effectiveness in retarding the growth of filamentous fungi. Only little information is available on its use to delay food spoilage due to bacterial growth [7]. In one of the first scientific studies, by Oh et al. [8], ethanol was found to inhibit the growth of many bacteria, as well as yeasts and filamentous fungi, in common laboratory culture media that had been spiked with 9% (v/v) ethanol (7.2 %, w/w).

The aim of this study was to determine the extent of inhibition of acetic bacteria by ethanol and ammonium bisulfite.

The results provided insights into how various concentrations of the aforementioned inhibitors in the medium are able to eliminate various species of acetic acid bacteria.

## MATERIALS AND METHODS

### Studied microorganisms

One representative of acetic acid bacteria from the genus *Gluconobacter* and one from the genus *Acetobacter* were selected for this research. The first species was *Gluconobacter oxydans* CCM 3618 (ATCC 33280) and the second was *Acetobacter aceti* CCM 3620$^T$ (ATCC 15973). Both bacterial species came from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic).

### Inoculation of microorganisms

Bacterial cultures in the form of gelatin discs were first transferred into Erlenmeyer's flasks containing Nutrient broth 1 (Himedia, Mumbai, India) and then the flasks were covered with Parafilm M (Sigma Aldrich, St Louis, Missouri, USA). The microorganisms were cultured for 24 h at 25 °C for *G. oxydans* and at 30 °C for *A. aceti*. Subsequently bacterial cultures were diluted with Nutrient broth 1 to a turbidity of 1 McF. The inoculum prepared in this manner was used for both methods.

### Plating methods

The disc diffusion method was chosen to investigate the inhibitory properties of ammonium bisulfite. This inhibitor was selected for its antioxidant, antibacterial and antifungal effects. This sulfur product (Esseco, Trecate, Italy), which contains no potassium but provides a source of nitrogen, is used to sulphurize musts and wines. To investigate the second inhibitor, ethanol (Lach-Ner, Neratovice, Czech Republic), it was injected directly into the inoculum, which was then spread evenly on the culture medium. This method was selected because of the volatile nature of ethanol. During the research, we optimized which concentrations would be most suitable. For this method, concentrations of ammonium bisulfite ranging from 0–100 $g \cdot l^{-1}$ were chosen, and to investigate the inhibitory properties of ethanol against the selected bacteria, concentrations ranging from 0 % to 48 % (v/v) were chosen for this method.

### Disk diffusion method

The standardized bacterial suspension in nutrient broth was used for inoculation of the agar medium. Volumes of 300 $\mu$l of the bacterial suspension with a turbidity of 1 McF were spread on a sterile Petri dish with Mueller Hinton agar (Himedia). Then, various concentrations of ammonium disulfite were applied to 6 mm diameter Sterile paper discs (Himedia). The following

concentrations of ammonium disulfite were used: 100 $g \cdot l^{-1}$, 50 $g \cdot l^{-1}$, 25 $g \cdot l^{-1}$, 12.5 $g \cdot l^{-1}$, 6.25 $g \cdot l^{-1}$, 3.125 $g \cdot l^{-1}$ and the control group 0 $g \cdot l^{-1}$ (distilled H2O). The prepared disks were first dried for a few minutes and then transferred to an agar plate with inoculum spread on it. The dishes with inoculum and impregnated discs were incubated in a thermostat at a temperature appropriate to the microorganism for 24 h at 25 °C for *G. oxydans* and at 30 °C for *A. aceti*. The diameters of the inhibition zones were then read.

#### Application of the inhibitor to the inoculum

The inhibitory properties of ethanol against acetic acid bacteria were also tested using the plating method. Due to the volatile nature of this substance, the method of applying the inhibitor directly to the inoculum, which was then spread evenly on the culture medium, was used. This involved dilution with six concentrations of ethanol (48 %, 24 %, 12 %, 6 %, 3 % and 1.5 % (v/v)) and 0 % (v/v) as a control group. Furthermore, 250 $\mu$l of the standardized bacterial suspensions were mixed with 50 $\mu$l of the above concentrations of the test substance. The seventh suspension was the control, where 50 $\mu$l of ethanol was replaced by 50 $\mu$l of distilled water. The inocula thus prepared were spread evenly on the surface of the culture medium. The plates were then left for 10 min in a laminar flow cabinet to allow complete absorption of the inoculum into the culture medium. Subsequently the samples were transferred to the incubator for 24 h, after which time the percentage of inhibited area was determined.

#### Monitoring growth curves

The growth curve method was also chosen to quantify the effect of both ammonium bisulfite and ethanol on the growth intensity of the studied bacteria. After several optimizations, the following concentrations were selected for this method. In the case of ammonium bisulfite, concentrations 0–12.5 $g \cdot l^{-1}$ were selected. Higher concentrations in each measurement resulted in devitalization of all bacteria in the inoculum. When testing the sensitivity of the selected acetic acid bacteria to ethanol, concentrations 0–96 % (v/v) were selected. An automated Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Boston, Massachusetts, USA) supplemented with SkanIt software for microplate readers (Thermo Fisher Scientific) was used to construct growth curves. Plastic 96-well microtitration plates were used for the measurements. First, the bacterial culture in the form of gelatin discs was transferred with a sterile needle into Erlenmayer flasks, together with sterile Nutrient Broth No. 1, and all flasks were covered with Parafilm M. The culture took place at shaking at frequency of 2.5 Hz for 24 h at 25 °C for *G. oxydans* and at 30 °C for *A. aceti*. The growth of microorganisms in the laboratory glass was easily detectable with the naked eye, as there was a strong turbidity of the otherwise clear broth. Subsequently, the bacterial suspension was diluted using sterile Nutrient Broth No. 1 to a turbidity level of approximately 1 McF. This culture was pipetted into the microtitration plates utilizing 270 $\mu$l of the diluted culture and 30 $\mu$l of distilled water as a blank. Into other wells, 270 $\mu$l of the diluted culture and 30 $\mu$l of various concentrations of the investigated inhibitors were pipetted. The microplates were loaded into the Multiskan FC photometer and the measurements were carried out for 24 h. The individual absorbance values were measured at regular intervals of 15 min, with the measurement wavelength set to 595 nm. Pulse shaking of the microplates was used between each measurement and the results were tabulated by the SkanIt software. The demonstrative results of the growth curves in this paper are expressed from a single measurement and the data were then plotted in Excel Pro Office 365 (Microsoft, Redmond, Washington, USA).

## RESULTS AND DISCUSSION

The standard diffusion disc method was first tried to test the inhibitory effects of both ammonium disulfite and ethanol. This method was only suitable for testing ammonium disulfite. Testing the inhibitory properties of ethanol against acetic acid bacteria using the disk diffusion method was excluded from this study due to the volatility of the inhibitor. Instead, the method of applying ethanol directly to the inoculated medium was used. In this research, the plating methods were further enriched by monitoring the growth curves of the bacteria in the presence of various concentrations of the selected inhibitors.

#### Application of ammonium bisulfite to cellulose discs

The inhibitory properties of ammonium bisulfite against both representatives of selected acetic acid bacteria were investigated by this method. The applied concentrations and inhibition zone sizes for *A. aceti* (CCM 3620$^T$) and *G. oxydans* (CCM 3618) are shown in Fig. 1. Concentrations from 3.125 $g \cdot l^{-1}$ to 100 $g \cdot l^{-1}$ ammonium bisulfite were used in this method. Distilled water was used as a control and did not show inhibitory

properties in either replicate. Here, we could see the inhibitory capabilities of ammonium bisulfite. In this method, *A. aceti* was found to be much more resistant to the antibacterial effects of the inhibitor than *G. oxydans*. The results showed that even a low concentration of the test substance (3.125 $g \cdot l^{-1}$ $(NH_4)HSO_4$) was able to inhibit the growth of *G. oxydans*. In contrast, the same inhibitory effect was obtained for *A. aceti* only at a concentration of 50 $g \cdot l^{-1}$ $(NH_4)HSO_4$. In *A. aceti*, the inhibition zones were often overgrown with colonies of resistant bacteria. In *G. oxydans*, such growths of colonies of resistant bacteria did not occur.

The zones demonstrated the inhibitory abilities of the tested ammonium bisulfite against *A. aceti* (Fig. 2). However, the results showed that this bacterium can live and actively multiply in an environment where high concentrations of ammonium bisulfite are present. As shown in Fig. 1, only a high concentration (100 $g \cdot l^{-1}$ $(NH_4)HSO_4$) was able to significantly inhibit the growth of the bacterium. When the concentration of the substance was halved, the ability of the inhibitor to prevent the growth of this bacterium was rapidly reduced. The other concentrations were unable to significantly inhibit the growth of the test microorganism. Distilled water was used as a control and showed no inhibitory properties.

The zones demonstrated strong inhibitory abilities of ammonium bisulfite against *G. oxydans* (Fig. 3). These results confirmed that the bacterium is more sensitive to this inhibitor than the previously tested representative. Fig. 2 shows that even a low concentration (6.25 $g \cdot l^{-1}$ $(NH_4)HSO_4$) could significantly inhibit the growth of the studied bacterium. A gradual decrease in the concentration of the substance resulted in only a slight reduction in the ability of this inhibitor to limit the growth of the acetic acid bacterium in question. Distilled water was used as a control and, again, it showed no inhibitory properties.

**Application of ethanol directly into the inoculum**

Antimicrobial properties of ethanol against acetic acid bacteria were also confirmed by this method. The concentration of ethanol chosen for testing was from 1,5 % to 48 % (v/v). A sta-

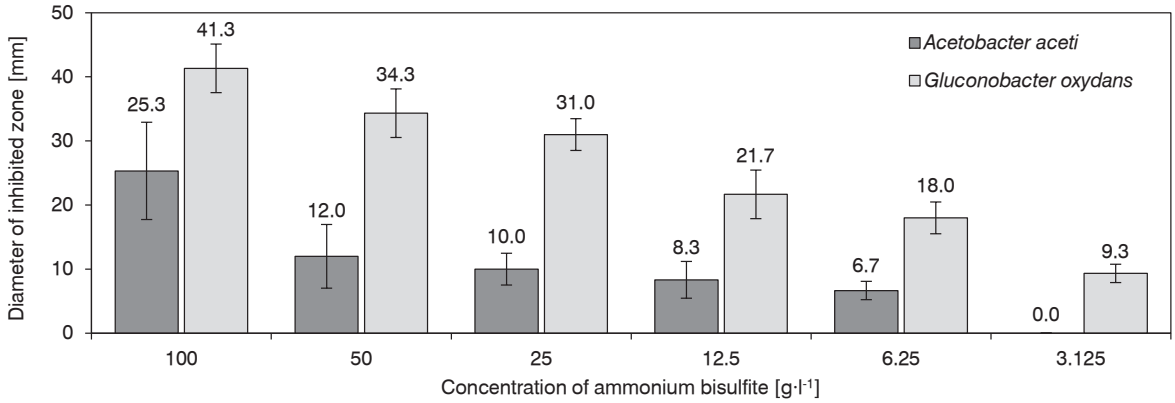


**Fig. 1.** Inhibition of *Acetobacter aceti* and *Gluconobacter oxydans* by ammonium bisulfite on nutrient agar.

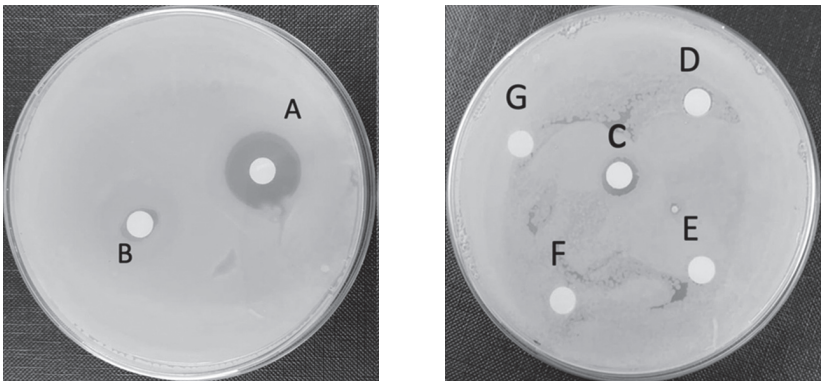


**Fig. 2.** Inhibition of *Acetobacter aceti* by ammonium bisulfite on nutrient agar.

Concentration of ammonium bisulfite: A – 100 $g \cdot l^{-1}$, B – 50 $g \cdot l^{-1}$, C – 25 $g \cdot l^{-1}$, D – 12.5 $g \cdot l^{-1}$, E – 6.25 $g \cdot l^{-1}$, F – 3.125 $g \cdot l^{-1}$, G – 0 $g \cdot l^{-1}$.

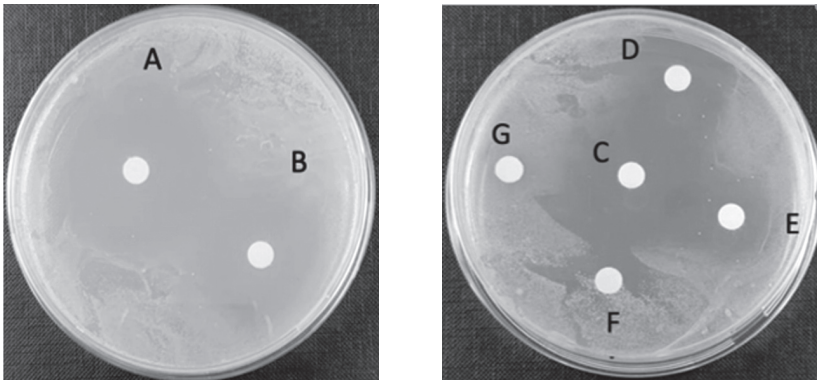


**Fig. 3.** Inhibition of *Gluconobacter oxydans* by ammonium bisulfite on nutrient agar.
Concentration of ammonium bisulfite: A – 100 g·l$^{-1}$, B – 50 g·l$^{-1}$, C – 25 g·l$^{-1}$, D – 12.5 g·l$^{-1}$, E – 6.25 g·l$^{-1}$, F – 3.125 g·l$^{-1}$, G – 0 g·l$^{-1}$.

tistical representation of the results is shown in Fig. 4. In this graph, higher resistance of *A. aceti* to the tested inhibitor compared to *G. oxydans* can be seen. The different extent of the growth of the colonies of the tested bacteria can be seen in Fig. 5 and Fig. 6. In the variants with lower ethanol concentration, both bacteria were able to completely fill the surface of the Petri dish.

In this method, the most pronounced difference in sensitivity to ethanol between the acetic acid bacteria in question was mainly at a concentration of 48 % v/v. In this case, *G. oxydans* showed high sensitivity to ethanol. On the other hand, *A. aceti* was able to fill the plate surface to 80 % even at this higher concentration. A gradual decrease in the concentration of the test substance led to a sharp increase in the growth of both bacteria. A dose of 6 % (v/v) was no longer able to prevent the growth of either bacterium. Distilled $H_2O$ was used as a control and showed no inhibitory properties.

**Growth curves in presence of ammonium bisulfite**

The inhibitory properties of ammonium bisulfite against the bacteria were also confirmed by this method. Fig. 7 and Fig. 8 show how ammonium bisulfite affected the growth of the microorganisms. Both graphs demonstrate the effectiveness of ammonium bisulfite against acetic acid bacteria at concentrations from 0 g·l$^{-1}$ to 12.5 g·l$^{-1}$.

According to Fig. 7, this method also confirmed the ability of ammonium bisulfite at certain concentrations to limit the growth of *A. aceti*. The measurements showed that higher concentrations of $(NH_4)HSO_4$ also limited the growth of this bacterium. All bacteria were devitalized when 30 $\mu$l of 12.5 g·l$^{-1}$ $(NH_4)HSO_4$ was added to the well of the microtitration plate. This was evidenced by the absorbance value measured after 24 h of cultivation, which was identical to the absorbance measured at the beginning of the experiment. There was some growth of bacteria when the inhibitor was diluted to half. However, the growth was clearly lower

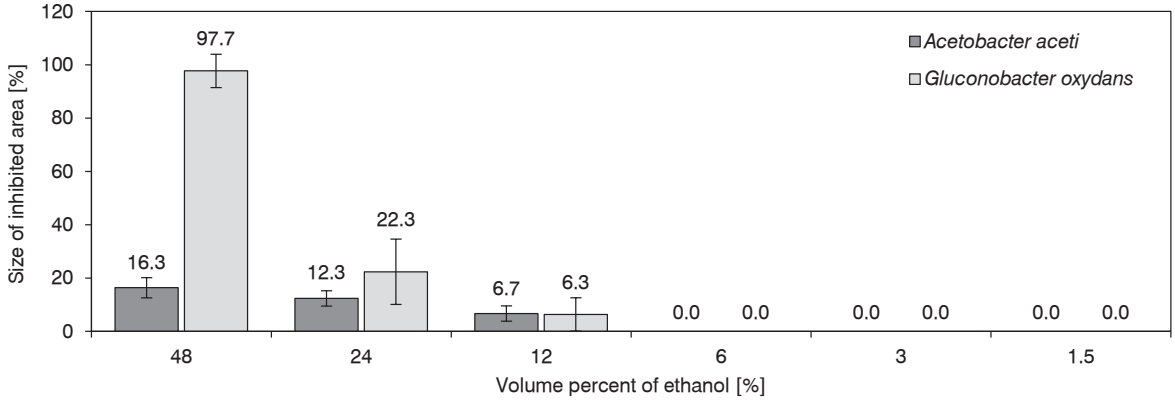


**Fig. 4.** Inhibition of *Acetobacter aceti* and *Gluconobacter oxydans* by ethanol on nutrient agar.

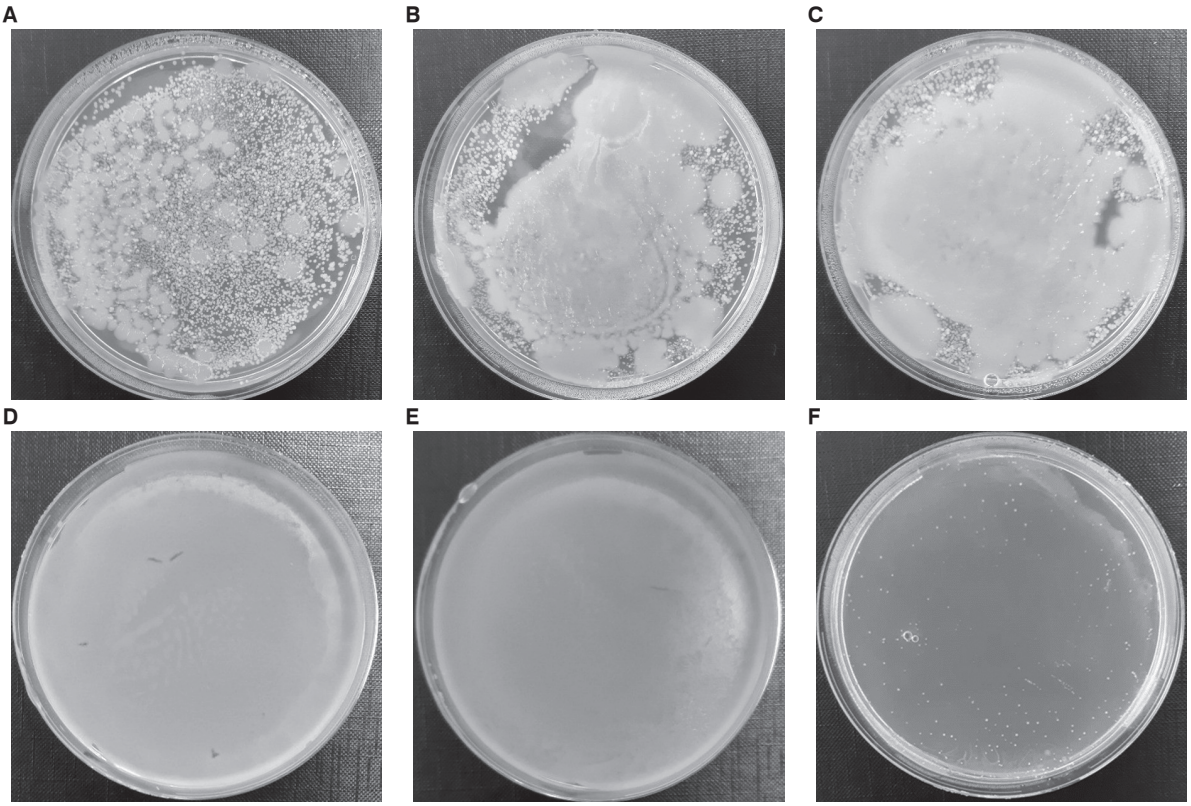


**Fig. 5.** Photographs of the results of inhibition of *Acetobacter aceti* by ethanol of various concentrations.

Volume percent of ethanol: A – 48 %, B – 24 %, C – 12 %, D – 6 %, E – 3 %, F – 1.5 %.

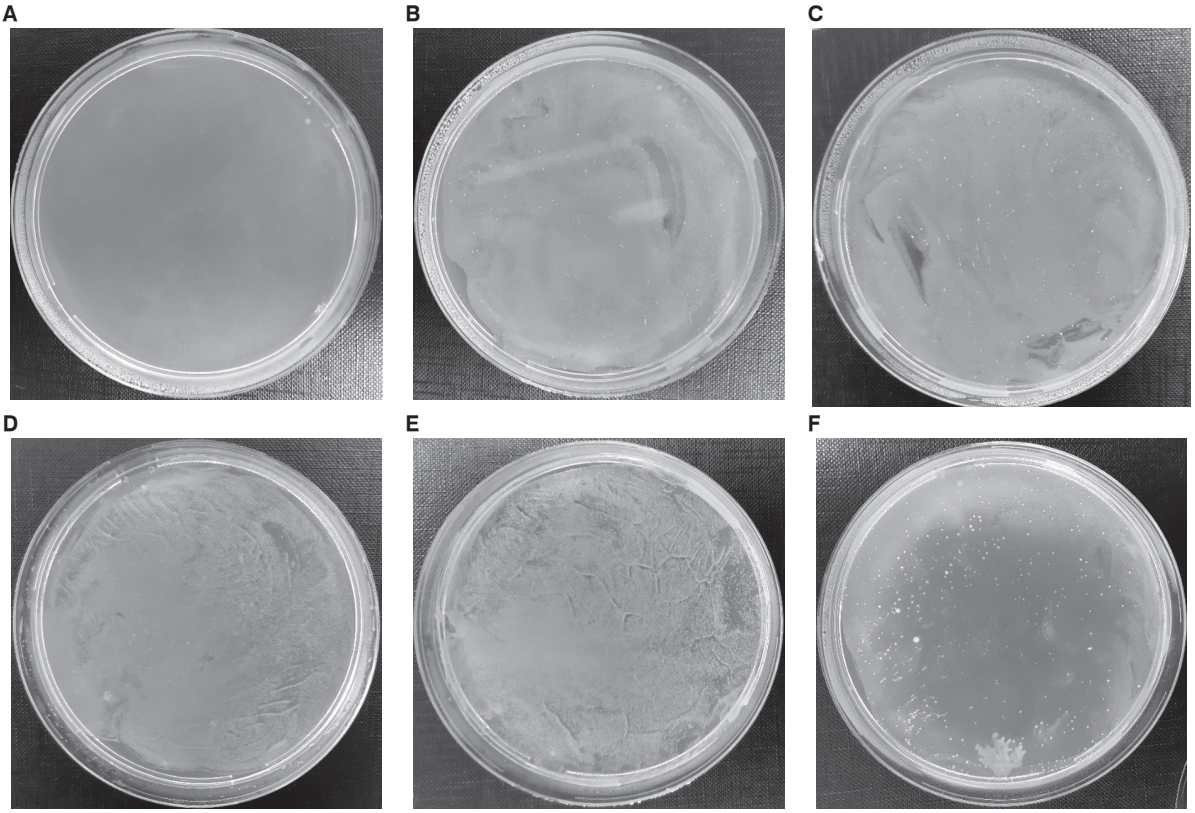


**Fig. 6.** Photographs of the results of inhibition of *Gluconobacter oxydans* by ethanol of various concentrations.

Volume percent of ethanol: A – 48 %, B – 24 %, C – 12 %, D – 6 %, E – 3 %, F – 1.5 %.

than in the control, when 30 μl of distilled $H_2O$ was added to the well instead of the inhibitor. The graph shows that not only did $(NH_4)HSO_4$ at concentrations of 1.56 g.l$^{-1}$ and 0.78 g.l$^{-1}$ not significantly limit the growth of the examined bacteria, but it even slightly promoted the growth of these microorganisms.

Fig. 8 shows the inhibitory ability of ammonium bisulfite against *G. oxydans*. All applied concentrations showed some inhibitory activity. Water did not inhibit the growth of this bacterium in any way. It can be seen from the graph that $(NH_4)HSO_4$ at concentrations of 12.5 g·l$^{-1}$ and 6.25 g·l$^{-1}$ completely inhibited the growth of this microorganism. When the concentrations of 6.25 g·l$^{-1}$ and 3.13 g·l$^{-1}$ were applied, there was already a slight increase in the bacteria in the well of the microtitration plate. However, the increase was not significant compared with the control. From the values of the growth curves, it can be concluded that a gradual decrease in the concentration of the investigated inhibitor led to a proportional increase in the growth of bacteria.

This method demonstrated that *A. aceti* was significantly more resistant to the inhibitor ($(NH_4)HSO_4$) than *G. oxydans*. This bacterium was unable to grow at an inhibitor concentration of 6.25 g·l$^{-1}$, while *A. aceti* grew this concentration. Similar results were also obtained with the disc diffusion method. This also confirmed that *G. oxydans* was more sensitive to the antimicrobial action of ammonium bisulfite.

**Growth curves in presence of ethanol**

The inhibitory properties of ethanol against the bacteria under study were also confirmed by this method. From Fig. 9 and Fig. 10, it can be concluded that ethanol at 0–96 % (v/v) affected the growth of the microorganisms. Fig. 9 shows that ethanol in higher concentrations could limit the growth of *A. aceti*. The inhibitory properties of ethanol at concentrations of 6–96 % (v/v) were evident, since lower absorbance values were measured for all these concentrations than for the control. In contrast, concentrations of 0.8–3 % (v/v) promoted the growth of this microorganism. The growth of the bacteria in the wells of the microtitration plate with distilled water (control) was not as intense as the growth in the presence of lower concentrations of ethanol.

Fig. 10 shows that ethanol at higher concentrations could limit the growth of *G. oxydans*. Like the previous bacterium, the inhibitory action of ethanol at concentrations of 6–96 % (v/v) were evident, as lower absorbance values were measured for all the concentrations mentioned than for the control. In contrast, ethanol at concentrations of 0.8–3 % (v/v) promoted the growth of this microorganism. The growth of bacteria in the wells of the microtitration plate with distilled water (control) was not as intense as the growth in the presence of lower concentrations of ethanol.

This method also showed that *A. aceti* was more resistant to ethanol than *G. oxydans*. Both bacteria showed identical growth promotion following application of ethanol at of 0.8–3 % (v/v). At these concentrations, the measured absorbance was higher than for the control. However, the difference in resistance between these bacteria can be found in the growth curves. *A. aceti* was capable of limited growth following the application of 30 μl of ethanol at 96 % (v/v) and 48 % (v/v) to the well of

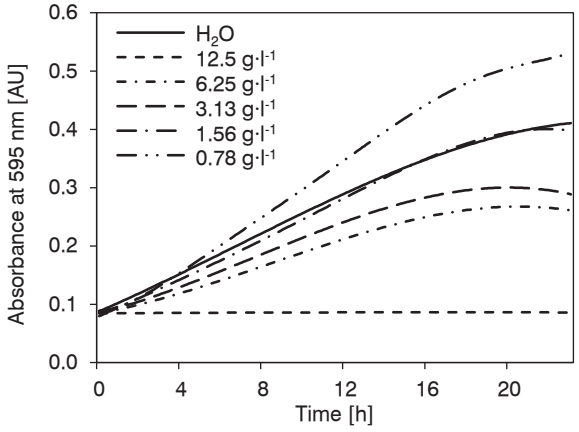


**Fig. 7.** Growth curves of *Acetobacter aceti* in the presence of various concentrations of ammonium bisulfite.

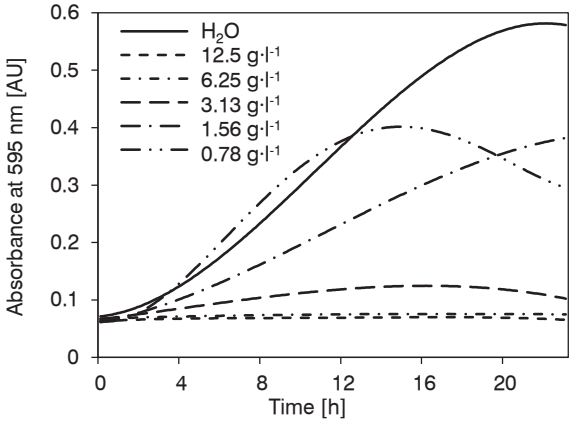


**Fig. 8.** Growth curves of *Gluconobacter oxydans* in the presence of various concentrations of ammonium bisulfite.

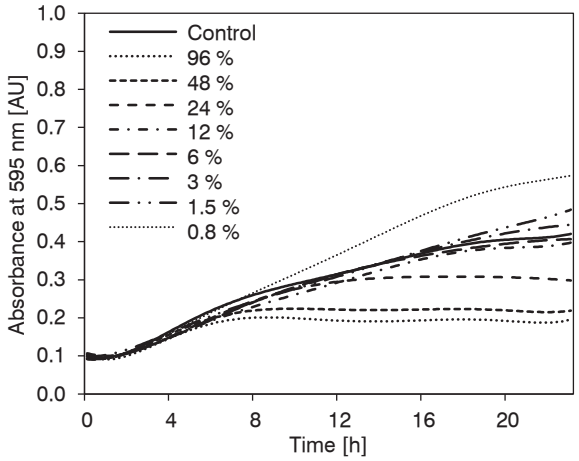


**Fig. 9.** Growth curves of *Acetobacter aceti* in the presence of various volume percent of ethanol.

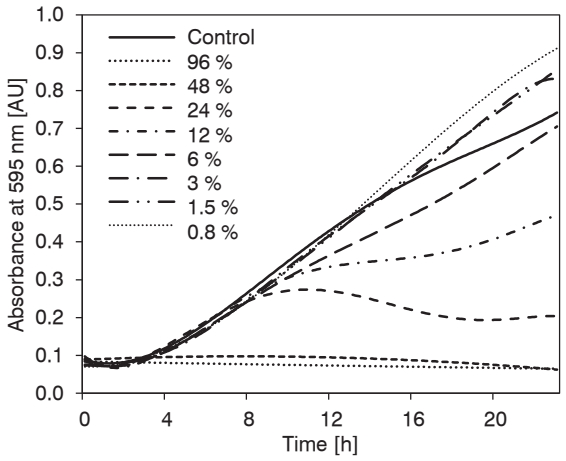


**Fig. 10.** Growth curves of *Gluconobacter oxydans* in the presence of various volume percent of ethanol.

the microtitration plate. At both concentrations, total inhibition for *G. oxydans* was caused.

The higher resistance of *A. aceti* to various types of inhibition was also reported in a study by KADERE et al. [9] who found that *G. oxydans* was more susceptible to extreme temperature fluctuations than *A. aceti*. That study was mainly concerned with the occurrence and identification of genera of acetic acid bacteria in coconut wine. All *Acetobacter* strains could oxidize ethanol to acetic acid and, finally, to $CO_2$ and $H_2O$, while the *Gluconobacter* strains could only oxidize ethanol to acetic acid as an end product. Both *Acetobacter* and *Gluconobacter* strains consistently grew at 25 °C, 30 °C and 40 °C as well as at pH 7.0 and pH 4.5, while no growth occurred at 45 °C, pH 2.5 and pH 8.5. *Acetobacter* strains oxidized both lactate and acetate, while *Gluconobacter* oxidized only lactate. Strains of both genera could ferment arabinose, xylose, ribose, glucose, galactose, mannose and melibiose. *Acetobacter* and *Gluconobacter* strains isolated in that study were found to be responsible for the spoilage of mnazi.

Some publications reported that even very low doses of sulfites have an inhibitory effect on acetic acid bacteria. However, total inhibition only occurs at significantly higher concentrations, which was confirmed by this study. Thus, the low doses of sulfites used in wine technology may not provide sufficient protection. However, the antimicrobial efficacy of wine itself is well documented. Phenols, ethanol, the low pH of the wine, $SO_2$ and other components of the wine certainly play a major role. It is accepted that ethanol or $SO_2$ alone will never achieve the same antibacterial activity as the synergistic combination of the many components in wine. The antibacterial activity of the samples could not be related to their total phenolic and resveratrol content, antioxidant capacity, ethanol content or pH. The antimicrobial activity of complex solutions, such as intact wine, cannot be attributed solely to its phenolic or non-phenolic components, nor can the antimicrobial activity of wine be predicted on the basis of its individual components [10].

In a study on the effects of silver nanoparticles [11], *A. aceti* was again found to be more resistant to the inhibiting agent than *G. oxydans*. In that study, similar methods were used to test the substance. In particular, the method of application of the substance to a cellulose disc clearly showed that bacteria of the genus *Gluconobacter* were more sensitive to silver nanoparticles than bacteria of the genus *Acetobacter*.

It was shown that at a concentration of 25 $mg \cdot l^{-1}$ of free sulfur dioxide, acetic acid bacteria can initiate acetic fermentation in wine. Only a concentration of 100 $mg \cdot l^{-1}$ of total sulfur could stop their growth. It was found that a dose of 100 $mg \cdot l^{-1}$ of total sulfur caused the amount of acetic acid bacteria in the must to be reduced by half. Although the bacteria were in a viable state, they no longer threatened the development of wine disease, and a double concentration reduced the amount of bacteria to almost zero. It was also found that at higher wine pH, it was necessary to increase the concentration of $SO_2$, as the effects of $SO_2$ diminish with higher pH. For example, at pH 6, a concentration of at least 200 $mg \cdot l^{-1}$ of sulfites was found to be required to completely eradicate acetic acid bacteria [12, 13]. In this context, research was oriented towards the alternatives to $SO_2$ in wine. Most studies focused on methods capable of replacing $SO_2$ as the anti-

microbial agent. The current state of knowledge about chemical additives and the innovative physical techniques that have been proposed for this purpose was comprehensively reviewed and showed that the microbiological stability of wine can be achieved by other methods [14].

## CONCLUSIONS

How sensitive the various species of acetic acid bacteria are to ethanol and ammonium bisulfite has yet to be precisely defined. This study aimed to determine the exact concentrations of selected inhibitory substances against acetic acid bacteria that could completely prevent their growth, which causes the most serious diseases of wines. The results showed that the selected inhibitory substances have good antibacterial properties in higher concentrations against *A. aceti* and *G. oxydans*. On the other hand, the results showed that low concentrations of the inhibitors led to the promotion of the growth of both acetic acid bacteria. *A. aceti* was able to grow even after the application of higher doses of ethanol, while *G. oxydans* showed higher sensitivity to both selected inhibitors than *A. aceti*.

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