STUDY OF INHIBITION OF YEASTS, LACTIC AND ACETIC BACTERIA USING SILVER PARTICLES

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ABSTRACT
This paper deals with a study of the inhibition of microorganisms occurring in grape must and wine, using silver in the form of nanoparticles and colloidal solution. Pure cultures of yeasts Saccharomyces cerevisiae and Brettanomyces bruxellensis, lactic acid bacteria Lactobacillus brevis, Pediococcus damnosus and acetic acid bacteria Acetobacter aceti and Gluconobacter oxydans were used for the experiments. Attention was primarily focused on monitoring changes in carbohydrate processing, namely glucose, fructose, sucrose, maltose, mannoit, galactose, trehalose, and ß-glucosidase activity. These biochemical determinations have shown limitations in carbohydrate processing, particularly sucrose in yeasts, and fructose, glucose and sucrose in bacteria. The effects of silver have also been observed in natural microflora found in grape must from Chardonnay and Hibernal. Colloidal silver at concentrations 40, 70 and 100 ppm and silver nanoparticles at concentrations 70, 150 and 250 ppm were used for inhibition. A plate method was used to determine the number of viable colonies. With an increasing concentration of applied substances, the growth of both yeasts and bacteria was strongly inhibited, as indicated by the numbers of colonies cultivated from the must. Yeast growth was inhibited by the lowest concentration – (70 ppm) by up to 72% and bacterial growth by up to 75.5%.

Keywords: silver nanoparticles, colloidal silver, wine, yeast, lactic acid bacteria, acetic acid bacteria

INTRODUCTION
Owing to its antimicrobial properties (Mijndendoneks et al., 2013), silver has been a frequent subject of interest in recent decades. Numerous studies have been conducted to investigate the inhibitory effects of various forms of silver and the mechanisms of action against model pathogenic bacteria, most commonly Escherichia coli (Choi et al., 2018), Staphylococcus aureus (Kang et al., 2019), Pseudomonas aeruginosa (Salomoni et al., 2017), and also against yeast, the most common representative of which is Saccharomyces cerevisiae (Kudrinsky et al., 2014).

The number of studies dealing with the application of silver to wine microorganisms is negligible. Nevertheless, the inhibitory effects are observed with colloidal silver (Izquierdo-Cañas et al., 2012) as well as with silver nanoparticles (García-Ruiz et al., 2015).

In the process of fermentation, saccharides are utilised by yeasts in several steps. It begins with glycolysis, where hexoses are converted to pyruvate, which is further decarboxylated to acetaldehyde, and this is reduced to alcohol. Glucose is preferred to fructose for fermentation, but yeasts S. cerevisiae are capable of processing galactose, sucrose, maltose, trehalose, melibiose, raffinose, melizitose and starch (Zimmermann and Entian 1997). Lactic acid bacteria process carbohydrates into lactic acid, and eventually also ethanol, acetate and CO₂. LAB observed by us can use arabinose, fructose, glucose, maltose, mannose, melibiose, ribose, raffinose and sucrose (Berłowska et al., 2016; Atuña and Martínez-Anaya, 1993).

Acetic acid bacteria form acetic acid from ethanol, but they oxidise glucose to gluconic acid. However, the AABs used in this study also oxidise other carbohydrates – arabinose, fructose, sucrose, galactose, mannose, ribose, sorbose and xylose (De Ley et al., 1984). The ability to process particular saccharides depends not only on the genus but also on the species and strain of microorganisms.

The aim of this study is to confirm the inhibitory effects of silver particles in the form of a colloidal solution and nanoparticles against microorganisms typical for wine production – yeast, lactic acid bacteria and acetic acid bacteria.

MATERIALS AND METHODS

Microorganisms
Pure cultures of microorganisms in the form of lyophilisates were used in laboratory experiments. The microorganisms Saccharomyces cerevisiae (CCM 8191), Lactobacillus brevis (CCM 1815), Pediococcus damnosus (CCM 2465), Acetobacter aceti (CCM 3620T) and Gluconobacter oxydans (CCM 3618) were obtained from the Czech Collection of Microorganisms. Yeasts, Brettanomyces bruxellensis, were isolated from wine. The lyophilisates of the microorganisms were activated by adding 0.3 mL of distilled water, allowed to recover for 15 minutes, and then the suspensions were transferred to Petri dishes onto the appropriate agar. The suspensions thus prepared were grown at 25°C for 24 hours. Yeasts were cultured on GYP agar (peptone 10 g.L⁻¹, beef extract 5 g.L⁻¹, yeast extract 1 g.L⁻¹, dextrose g.L⁻¹, agar g.L⁻¹, HiMedia); lactic acid bacteria (LAB) were cultured on MRS agar (glucose 20 g.L⁻¹, ammonium hydrogen citrate g.L⁻¹, potassium hydrogen phosphate 2 g.L⁻¹, magnesium sulfate 0.1 g.L⁻¹, manganese sulfate 0.05 g.L⁻¹, meat extract 5 g.L⁻¹, sodium acetate 5 g.L⁻¹, peptone 10 g.L⁻¹, yeast extract 5 g.L⁻¹, agar 12 g.L⁻¹, HiMedia) and acetic acid bacteria (AAB) were cultured on Acetobacter agar (peptone 5 g.L⁻¹, yeast extract 5 g.L⁻¹, mannitol 25 g.L⁻¹, agar 15 g.L⁻¹, HiMedia).

Chemicals
Colloidal silver in concentration of 100 ppm (Rulcotherapy, Czech Republic) in the form of an aqueous solution, and silver nanoparticles in concentration of 250 ppm, with particle sizes ranging from 6 to 12 nm (Nano-BioTech, Poland). Lower concentrations of solutions were achieved by dilution with distilled water.

Determination of saccharides consumption and ß-glucosidase activity
30 ml of sterile 0.9% saline was pipetted into a conical flask, a sufficient amount of suspension was added. The resulting suspension was then pipetted into prepared microtiter plates with a desiccant (Erba Lachema, Czech Republic). 300 µL of suspension was placed in wells without the use of silver particles (control) or 290 µL of suspension and 10 µL of silver particles. Plates thus prepared, were loaded into a photometer.
(Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, USA) and the individual absorbances were measured. Wavelengths varied based on the used test, i.e. 595 nm for yeasts (CandidaScreen kit) and 405 nm for LAB and AAB (ANAEROtest 23 kit for LAB and NEFERMtest 24 kit for AAB). The temperature during the measurement was 30°C, the number of measurements was 96, with a measurement interval of 15 minutes and with shaking for 20 seconds at the beginning of the interval.

Basic must analysis

Before fermentation, basic characteristics were determined – sugar amount using a digital refractometer (Atago, Japan), pH using a laboratory pH meter (inoLab® pH 7110, WTW, Czech Republic), titratable acids content, and yeast assimilable nitrogen (YAN) with an ALPHA II automatic analyser (Bruker, USA).

Determination of microbial stability of must

500 ml of Hibernal and Chardonnay grape must was transferred to conical flasks and 10 ml of silver nanoparticles at 10, 70, 150 or 250 ppm or colloidal silver at 10, 40, 70 or 100 ppm were added to all flasks (except the control). After 24 hours at 20°C, all variants were shaken and diluted 10,000 x in tubes (1 µL of must was added to 9.999 mL of sterile 0.9% saline). To determine microbial stability, the prepared suspensions were applied to selective agars – 1 mL of each variant was transferred to GKCH agar (chloramphenicol prevented bacterial growth), MRS agar (for LAB growth) and Acetobacter agar (for AAB growth). After 48 hours of cultivation, grown colonies were counted.

RESULTS

The results are divided into two parts. In the first part are presented absorbance changes in carbohydrate processing, in the second part microbial stability is studied.

Biochemical determination

Monitoring of carbohydrate consumption and β-glucosidase activity in studied microorganisms was performed by observing absorbance changes. From the results of this determination, it can be concluded that the processing of saccharides is influenced, especially in bacteria (LAB and AAB), but the yeasts are not much affected. Figure 2 shows the absorbance values of the studied cultures after 24 hours.

Figures 1 and 2 show changes in carbohydrate processing over time in yeast S. cerevisiae and B. bruxellensis, both under the action of colloidal silver and AgNPs. The observed changes were in the fermentation intensity of trehalose, galactose, maltose and sucrose, due to the ability of selected yeasts to ferment these carbohydrates. In the case of S. cerevisiae (Figure 1), there were visible changes in maltose processing. There was a clear inhibition using both colloidal silver and AgNPs. For B. bruxellensis (Figure 2), both types of silver particles had visibly altered the course of fermentation only with sucrose.

L. brevis, respective its metabolic processes, was inhibited by both types of silver particles used (Figure 3). Compared with controls, there was a change in all carbohydrates. Control samples showed an increase in absorbance, whereas the variants using colloidal silver and AgNPs tended to decrease due to the strong inhibition caused by the application of silver particles. The inhibition rate corresponds with the concentration of silver particles applied.
Figure 4 Absorbance changes in carbohydrate processing by P. damnosus bacteria over 24 hours depending on the concentration of colloidal silver and AgNPs. (A) fructose, (B) glucose, (C) maltose, (D) sucrose.

A representative of lactic acid bacteria, P. damnosus, was inhibited by all concentrations of both silver particles (Figure 4). The rate of inhibition of silver nanoparticles was concentration-dependent – the higher the concentration, the more the processing was limited. In contrast, colloidal silver inhibition was not concentration-dependent – the lowest concentration used avoided processing of all carbohydrates almost identically to the highest concentration.

Figure 5 Absorbance changes in carbohydrate processing by A. aceti bacteria over 24 hours depending on the concentration of colloidal silver and AgNPs. (A) mannitol, (B) sucrose, (C) β-glucosidase activity, (D) galactose.

In Figure 5, we can see the effect of silver particles on acetic acid bacteria A. aceti, where processing of mannitol, sucrose, galactose, and β-glucosidase enzyme activity were observed. All processing and β-glucosidase activity were inhibited by both colloidal solution and nanoparticles. All controls showed an increase in absorbance, i.e. an increase in cell mass. Inhibition was the most pronounced at the highest concentrations of both particle types. Nevertheless, the differences in inhibitory effects among concentrations were not significant in this instance, given the absorbance values of the controls.

Figure 6 Absorbance changes in carbohydrate processing by G. oxydans bacteria over 24 hours depending on the concentration of colloidal silver and AgNPs. (A) mannitol, (B) sucrose, (C) β-glucosidase activity, (D) galactose.

The second AAB representative G. oxydans showed the same results as A. aceti. Again, all carbohydrate processing and β-glucosidase activity were inhibited, with differences in concentrations in terms of inhibition rates being minimal, as can be seen in Figure 6.

Determination of microbial stability

The growth of colonies of yeasts, lactic and acetic bacteria, naturally present in must and often undesirable, was observed. Both types of particles were compared to the control and the results show significant inhibitory effects. Results are expressed as the average of three measurements.

Must was analysed for basic must parameters (sugar amount, pH, total acidity and yeast assimilated nitrogen - YAN) and the measured values are shown in Table 1.

Table 1 Basic must parameters

| Parameter                | Value       |
|--------------------------|-------------|
| Sugar amount             | 15.8 °NM    |
| pH                       | 3.62        |
| Total acidity            | 6.0 g.L⁻¹   |
| YAN                      | 417.0 mg.L⁻¹|
The known inhibitory effect of silver was confirmed in this study by two methods – applying the particles to the must to inhibit the natural microflora and then determining the number of viable colonies, and biochemical determination of carbohydrate processing by pure cultures of yeasts (S. cerevisiae, B. bruxellensis), LAB (L. brevis, P. damnosus) and AAB (A. aceti, G. oxydans).

**DISCUSSION**

Owing to its antimicrobial effects, silver is still a frequent subject of research (Oves et al., 2018; Pugazhendhi et al., 2018; Shannuganathan et al., 2018). Given the presence of a wide range of microorganisms in grape must and wine and the lack of studies on this topic, this research has explored the possibility of inhibiting microorganisms using silver and eliminating their effects on wine. After the application of colloidal silver to the must, both yeasts and lactic and acetic acid bacteria contained in the must were inhibited. The most sensitive to silver nanoparticles appears to be lactic acid bacteria, which were absolutely inhibited by the three highest concentrations (70, 150 and 250 ppm). However, even the lowest concentration, 10 ppm, reduced growth by 62%, so was effective compared with the colony growth of the control. In contrast, acetic acid bacteria were the least sensitive to AgNPs in this experiment. Even at the highest concentration, their growth was not completely ceased. The lowest concentration, 10 ppm, limited growth by only 11%. It was also the least represented group of microorganisms in the grape must. As the concentration increased, the inhibitory power of both types of silver particles also increased, with colloidal silver generally showing stronger inhibitory effects.

The inhibitory effect of silver was confirmed in this study by two methods – applying the particles to the must to inhibit the natural microflora and then determining the number of viable colonies, and biochemical determination of carbohydrate processing by pure cultures of yeasts (S. cerevisiae, B. bruxellensis), LAB (L. brevis, P. damnosus) and AAB (A. aceti, G. oxydans).

**CONCLUSION**

This study focused on the inhibitory effect of colloidal silver and silver nanoparticles against yeasts, lactic and acetic acid bacteria, both in the forms of pure cultures and wild, naturally occurring cultures. The aim of this study was to monitor the effects of silver particles and confirm their possible use as an inhibitory agent. Results of the methods we applied confirmed these effects, the silver inhibited the diverse natural grape must culture, but also representatives of lactic and acetic acid bacteria in the form of pure cultures. The only problematic species seemed to be pure culture yeasts, for which biochemical determination did not support this hypothesis. An important aspect of the use of silver is its negative impact on human health, thus, it is desirable to focus on the possibility of its removal from wine and long-term exposure to the small concentrations required to inhibit microorganisms in future studies.

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