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

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INTRODUCTION

Owing to its antimicrobial properties (Mijendonecks 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 aeruginos (Salomoni et al., 2017), and also against yeast, the most common representative of which is Saccharomyces cerevisiae (Kudrinskly 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-Cañás 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 dehydroxyylated 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, melezitose and starch (Zimmermann and Entian 1997). Lactic acid bacteria process carbohydrates into lactate, and eventually also ethanol, acetate and CO₂. LAB observed by us can utilise arabinose, fructose, glucose, maltose, mannnose, melibiose, ribose, raffinose and sucrose (Berłowska et al., 2016; Atuña and Martinez-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, mannnose, 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 lyophilizates 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, yeast extract 5 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 (meat 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) was used 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 β-glucosidase activity

30 ml of sterile 0.9% saline was pipetted into a conical flask, a sufficient number of microorganisms (to a degree of turbidity of 2 McF, about 6x10⁸ CFU/ml) were transferred into the solution with a sterile loop and mixed well. 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

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Microbiol Biotech Food Sci / Chvalinova et al. 2021 : 10 (4) 581-585

(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.

![Figure 1 Absorbance changes in carbohydrate processing by S. cerevisiae yeast over 24 hours depending on the concentration of colloidal silver and AgNPs. (A) trehalose, (B) galactose, (C) maltose, (D) sucrose.](image1)

![Figure 2 Absorbance changes in carbohydrate processing by B. bruxellensis yeast over 24 hours depending on the concentration of colloidal silver and AgNPs. (A) trehalose, (B) galactose, (C) maltose, (D) sucrose.](image2)

![Figure 3 Absorbance changes in carbohydrate processing by L. brevis bacteria over 24 hours depending on the concentration of colloidal silver and AgNPs. (A) fructose, (B) glucose, (C) maltose, (D) sucrose.](image3)

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

<table>
<thead>
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<th>Sugar amount</th>
<th>pH</th>
<th>Total acidity</th>
<th>YAN</th>
</tr>
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<tbody>
<tr>
<td>15,8 °NM</td>
<td>3,62</td>
<td>6,0 g.L⁻¹</td>
<td>417,0 mg.L⁻¹</td>
</tr>
</tbody>
</table>

Table 1 Basic must parameters

<table>
<thead>
<tr>
<th>Number of CFU*10⁴/mL</th>
<th>Colloidal 100</th>
<th>Colloidal 70</th>
<th>Colloidal 40</th>
<th>Colloidal 10</th>
<th>Control</th>
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<td>Colloidal 100</td>
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<td>Control</td>
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S. cerevisiae
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teria. The highest concentration used in this study, 100 ppm, has no significant effect. Conversely, lactic acid and acetic acid bacteria are inhibited almost equally by all concentrations. Control curves showed an upward trend in almost all cases over 24 hours, which can be attributed to the multiplication of bacteria in the presence of individual markers (carbohydrates).

The curves of variants with silver particles have decreased in many cases. Apparently, there has been a change in colour, but not due to the processing of carbohydrates. This phenomenon may be due to a change in pH or a high cultivation temperature (30°C). Monitoring of metabolic pathways affected by the presence of metals is also a unique research topic (Oliveira et al., 2012; Das, 2012). Changes in carbohydrate concentrations have been confirmed by several studies. The action of antimicrobial agents, including silver nanoparticles, causes release of trehalose and glucose, both outside and inside Candida albicans

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 nanoparticles 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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REFERENCES