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Chitooligosaccharide as A Possible Replacement for Sulfur Dioxide in Winemaking

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Featured Application: Chitooligosaccharide (COS) can be easily obtained by hydrolyzing chitosan oligomers produced from crustacean shell waste. COS possesses a wide range of biological activities, including antibacterial, antifungal, antiviral, antitumor, antioxidant, immunoregulatory, blood pressure control, and hypocholesterolemic effects. In contrast, SO_2 might cause health problems, such as allergic reactions. Furthermore, excessive use of COS doesnot negatively affect wine quality. Therefore, this study focuses on investigating the antimicrobial activity of COS during winemaking in comparison with the action of SO_2. An economical amount (500 mg/L) of COS used as an additive during winemaking shows a comparable antimicrobial effect to 100 mg/L SO_2 and does not affect the fermentation process. Therefore, COS is a potential replacement for SO_2 in winemaking.

Abstract: Sulfur dioxide (SO_2) has been used for centuries as a preservative in winemaking. However, the addition of SO_2 is associated with allergic reactions and can negatively affect wine quality. In our work, chitooligosaccharide (COS) was applied as an alternative to SO_2 in winemaking, and its antimicrobial activity during winemaking was investigated in comparison with the action of SO_2. The optimal concentration of COS was identified as 500 mg/L. The antimicrobial effect of COS was evaluated using known and our own separated wine spoilage organisms. The antimicrobial effect of 500 mg/L COS was found to be comparable with that of 100 mg/L SO_2. Furthermore, using 500 mg/L COS as an additive during winemaking did notinfluence the cell growth of *Saccharomyces cerevisiae*. Therefore, COS can be used as an additive in winemaking.

Keywords: sulfur dioxide; chitooligosaccharide; antimicrobial effect; winemaking; wine spoilage organism

1. Introduction

Sulfur dioxide (SO_2) has been used in winemaking for centuries as a versatile and efficient additive owing to its antimicrobial and antioxidant properties. SO_2 is often added to a grape crusher prior to fermentation to control unwanted microorganisms and polyphenol oxidase activity during winemaking, and it is added to wine after malolactic fermentation and prior to bottling as a preservative agent. The total amount of SO_2 added throughout the winemaking process can vary considerably, but it is normally between 160 and 250 mg/L (ppm). A sufficient concentration of SO_2 should be supplied to ensure wine has adequate stability against excessive oxidation or microbial development, which can compromise wine quality. Du and Pretorius [1] confirmed that prohibiting SO_2 as an antimicrobial agent without a suitable alternative would increase the risk of wine spoiled by yeasts and bacteria. Nevertheless, owing to potential related health problems, the use of SO_2 inwine has

recently become a subject of focus. Sulfites resulting from the addition of SO_2 to wine have been associated with allergic reactions incurred by sulfite-sensitive individuals, who can experience a range of symptoms, including headaches, nausea, gastric irritation, and breathing difficulties in asthma patients [2]. Furthermore, excessive use of SO_2 can negatively affect wine quality, causing an organoleptic alteration of the final product that can result in unpleasant flavors and even produce characteristic aroma defects [3]. Consequently, reducing or even eliminating the use of SO_2 as a preservative and identifying safe alternatives to SO_2 preservation are crucial to provide a product that complies with winemaker demands without health-related problems.

As reported previously, ascorbic acid and sorbic acid can act as wine preservatives in conjunction with SO_2 because of their oxygen-scavenging abilities and capacities to reduce oxidized components [4,5]. The antimicrobial effects of ascorbic acid, bitartrate, sorbate, and citrate were less reported. Moreover, ascorbic acid was found to have pro-oxidant activity, which may promote browning coloration and enhance reductive off-odors of wine [4]. High concentration of sorbic acid (>400 mg/L) was also found to influence the quality of wine [5]. Tartaric acids were found to play an important role in wine oxidation, and potassium bitartrate may be precipitated from wine with inadequate cold stabilization [6,7]. Therefore, these organic acids or salts of organic acids are not always preferred as preservatives in wine or during winemaking.

Nowadays, many novel methods have been proposed for the substitution of SO_2, including adding compounds, such as dimethyl dicarbonate (DMDC) [8], bacteriocins [9], phenolic compounds [10], glutathione [11], and lysozyme [12], and physical methods, such as ultrasound, ultraviolet radiation, pulsed electric fields (PEF), and high hydrostatic pressure (HHP) [13–15]. Compared with the physical methods, the addition of compounds seems relatively versatile for facilities to use at different stages of winemaking. However, no reported methodology is suitable for completely replacing the use of SO_2, because SO_2 has a quite extensive conservation effect on wine. Therefore, alternative preservatives and innovative technologies that are nonharmful can substitute or at least complement the action of SO_2, allowing its concentration in wine to be decreased, whichremain in great demand [16].

Recently, chitooligosaccharide (COS) has been widely used in large-scale commercial applications and possesses multiple biological activities, including antimicrobial, antifungal, antiviral, antitumor, and antioxidant activities [17,18]. Owing to its antimicrobial and antioxidant activities, COS has been proposed as a food preservative in beers and foods of animal and aquatic origin [19–21]. COS added to various foods can not only preserve food quality and extend shelflife, but also act as a functional food ingredient and nutraceutical. Chitosan of fungal origin has already been verified as a new tool for controlling *Brettanomyces bruxellensis* in winemaking [22]. COS is more convenient to use than chitosan owing to its water solubility and low molecular weight. In a recent study, gallic-acid-conjugated COS was found to possess a high DPPH radical scavenging activity and a protective effect against H_2O_2-induced DNA damage [23]. This can inhibit the oxidation of wine constituents, including phenolics, certain metals, tyrosine, and aldehydes, during the winemaking process, while retaining the sensory and nutritional value of wine. Furthermore, as previously reported, adding COS-based materials can reduce the concentrations of metals, such as boron, lead, mercury, and antimony, and that of ochratoxin A in process water [24–27], which can markedly improve wine quality. Therefore, in this study, COS was applied as an alternative to SO_2 in winemaking, and the antimicrobial activity of COS during winemaking was investigated using our own separated contaminated organisms and known wine spoilage organisms in comparison with the action of SO_2. This is the first report of COS being applied as an alternative to SO_2 in winemaking. The results show that COS is a potential replacement for SO_2 in winemaking.

2. Materials and Methods

2.1. Must Preparation

To simulate winemaking, a must was prepared on a small scale using a glass fermentation tank rather than a stainless-steel fermentation tank. Three kilograms of Kyoho grapes purchased from a local market were used for must preparation. Grapes were destemmed, washed, squeezed with a household juicer and crushed. The squeezing time was set at only 2 s to ensure that the seeds were not broken. The resulting juice was filtered using a sterile canvas to eliminate solid impurities that remained in the juice. A filtrate was added into a sterile glass fermentation tank and pasteurized by boiling in a 72 °C waterbath for 30 min. After pasteurization, the resulting sample was cooled down by ice-water bath right away to circumvent the aging of flavor compounds. Finally, the samples were retained at 4 °C until used after being divided into several groups (200 mL each).

2.2. Isolation of Wine Spoilage Organisms and Culture Conditions

As microorganisms existing on the grape skin are more likely to resist ethanol and survive in rotten grapes compared with microorganism contaminants from the environment, fresh grapes were not washed and exposed to air to allow for decay. Once the fresh grapes exposed to air began to rot and the smell of ethanol was detected, they were picked out to prepare a must. The contaminated must was transferred to a fermentation flask and fermented at 25 °C for 10 days with shaking every 12 h.

After fermentation, bacteria were isolated by inoculating the fermentation broth in a beef extract–peptone agar medium with 0.0125% filter-sterilized nystatin (pH: 7.0–7.2). Yeasts were isolated by inoculating the fermentation broth on a yeast extract peptone dextrose (YEPD) agar medium with 32,000 U/L filter-sterilized penicillin (pH: 6.0). Fungi were isolated by inoculating the fermentation broth on the Czapek-Dox agar medium with 40,000 U/L streptomycin (pH: 7.2). A separated single colony was submitted to Comate Bioscience Company, Ltd. (Nanjing, China) for further strain identification by 16S rRNA gene sequencing or 18S rRNA gene sequencing.

2.3. Vinification

Food-grade COS was provided as a gift by Dalian GlycoBio Company, Ltd. (China). The COS average molecular weight was less than 2000 Da, and the degree of polymerization was in a range of 2–10. Before adding to a pasteurized must, various concentrations of SO_2 (50, 100, and 200 mg/L) in the form of potassium metabisulfite or those of COS (0, 100, 500, and 1000 mg/L) were filtered through a 0.22μm filter membrane. A must without any additive was used as a blank group. All samples were then inoculated with 0.25 g/L *Saccharomyces cerevisiae* (Angel, Yichang, China)and 2 mL/L wine spoilage organism (specific type or mixture). During fermentation, the temperature was kept at 25 °C, and the samples were shaken every 12 h for 10 days.

2.4. Colony-Counting Assay

After fermentation, appropriate dilutions of bacterial/fungal suspension were prepared. Each suspension (100 μL) was uniformly spread onto overnight-dried beef extract–peptone agar plates or Czapek-Dox agar plates with a sterile spatula. After incubation at 30 °C for 24 h, all colonies were enumerated, and the mean values and the maximal scatter in CFUs were determined.

2.5. Application of Known Spoilage Microorganisms to Vinification

Pediococcus damnosus, *Acetobacter aceti*, *Lactobacillus plantarum*, and *Lactobacillus brevis*, which are known to spoil musts and wines, were selected for further application. *Pediococcus damnosus* and *Acetobacter aceti* were purchased from Biobw (Beijing, China). *Lactobacillus plantarum* and *Lactobacillus brevis* were stored in our laboratory. A pasteurized must supplemented with SO_2 or COS was inoculated with *Saccharomyces cerevisiae* (0.25 g/L) and one known wine spoilage organism (2 mL/L).

During fermentation, the temperature was kept at 25 °C, and the samples were shaken every 12 h for 10 days.

As the spoilage abilities of the known spoilage microorganisms were different, a colony-counting assay was conducted every 24 h for 5 days. At each time point, the appropriately diluted bacterial suspension was uniformly spread onto overnight-dried *Lactobacilli* MRS broth agar plates or *Acetobacter* medium agar plates containing natamycin (0.13 g/L) with a sterile spatula.

2.6. Growing Curves of Saccharomyces Cerevisiae and PichiaPastoris

Pichiapastoris (stored in our laboratory) is a type of wild yeast that can cause wine spoilage. Therefore, the effects of COS on the cell growths of *Pichiapastoris* and *Saccharomyces cerevisiae* were investigated using growth curves. A pasteurized must supplemented with 500 mg/L COS was inoculated with 0.2 g/L *Saccharomyces cerevisiae* or 2 mL/L *Pichiapastoris*. A pasteurized must without added COS, but inoculated with the same amount of *Saccharomyces cerevisiae* or *Pichiapastoris*, was set as a blank group. During fermentation, the temperature was kept at 25 °C, and the samples were shaken at 100 rpm for 18 h. The bacterial concentration was determined every 2 h using the optical density (OD) value at 600 nm (OD_{600}).

2.7. Statistical Analysis

All colony-counting assays were performed in triplicate. Analysis of means and standard deviations were tested using SPSS software (version 17.0; IBM Corp., New York, USA).

3. Results and Discussion

As shown in Figure 1, wine spoilage organisms were first isolated from rotten grapes using a beef extract–peptone medium or Czapek-Dox medium. The supplemented concentration of COS was then optimized by investigating the antimicrobial effects of various concentrations of COS (0, 100, 500, and 1000 mg/L) on each type of wine spoilage organism. Furthermore, the antimicrobial effects of the optimized concentrations of COS and SO_2 were compared. Finally, the antimicrobial effects of COS on the known wine spoilage microorganisms were estimated, and the effects of COS on the cell growths of *Saccharomyces cerevisiae* and *Pichiapastoris*, a type of wild yeast that can cause winespoilage, were evaluated.

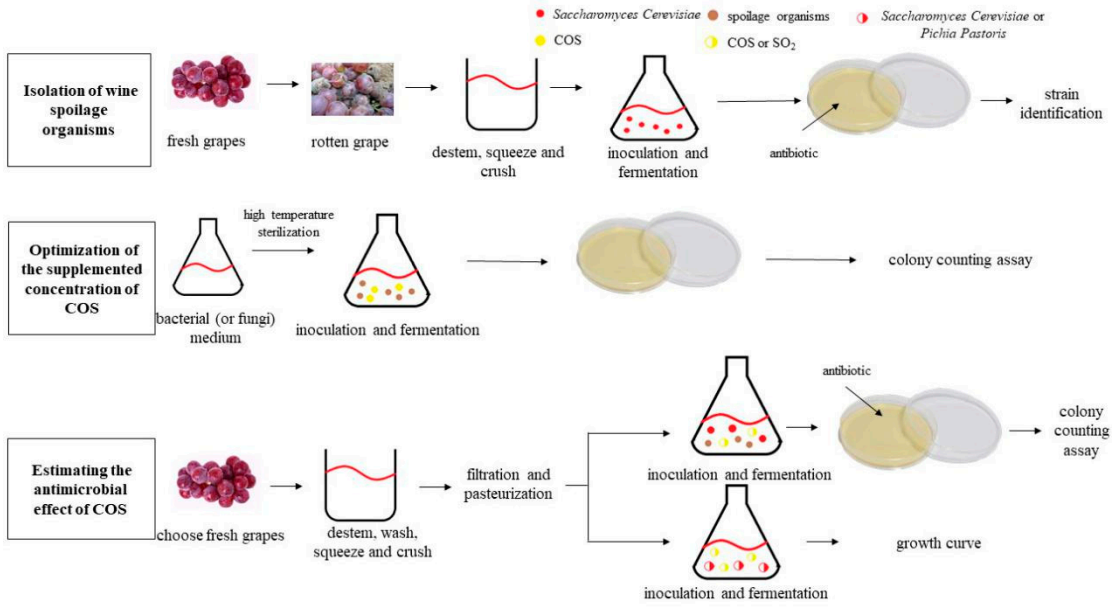

Figure 1. The workflow for investigation into whether chitooligosaccharide (COS) can be applied as an alternative to SO_2 in winemaking.

3.1. Optimization of Supplemented COS Concentration

Nine types of wine spoilage organism were isolated from the contaminated must, comprising six types of bacteria (*Enterococcus*, *Bacillus cereus*, *Paenibacillus*, *Alkaliphilus*, *Pantoea* sp., and *Lactococcus*) and three types of fungi (*Aspergillus flavus*, *Aureobasidium pullulans*, and *Penicillium*). Various concentrations of COS (0, 100, 500, and 1000 mg/L) were added into the beef extract–peptone medium or Czapek-Dox medium without agar and then inoculated with a specific type of wine spoilage organism to investigate the antimicrobial effect. The antimicrobial effect of COS was evaluated by measuring the colony counts of the nine types of spoilage organism when applying COS at 100, 500, and 1000 mg/L to the must. Many previous reports have described the antibacterial and antifungal activities of COS against many types of bacteria and fungi [28–31]. COS can alter the cell membrane permeability, which protects the release of cell components and controls the entry of materials into the cell from the environment. Positively charged COS can then bind to and be absorbed into microbe cell walls, causing DNA penetration and blocking RNA transcription and finally resulting in microbial cell death [30]. However, the antimicrobial activities of COS change depending on various factors, such as the degree of polymerization and microorganism type. Therefore, to efficiently exert the antibacterial activity of COS during winemaking, the COS concentration supplemented into the must should be optimized.

As shown in Table 1, when 100 mg/L COS was applied to the must, most wine spoilage organisms were inhibited to a certain extent, and the colony countswere decreased, which were ina range of 12%–95% of those without COS. The inhibition rate was calculated according to the following formula: inhibition rate = (colony count (with COS)–colony count (without COS))/colony count (without COS). *Enterococcus* and *Lactococcus* were only inhibited by 21% and 12%, respectively. When the COS concentration was increased to 500 mg/L, the inhibition rates of bacterium or fungus reached almost 100%. When the COS concentration was increased to 1000 mg/L, the colony count was further decreased by one or two orders of magnitude, but the inhibition rate was not markedly changed. By considering the economic benefits and the antimicrobial effect, 500 mg/L COS was selected as the optimized concentration.

Table 1. The colony counts of nine types of spoilage organisms when applying 100, 500, and 1000 mg/L COS in the must.

Wine Spoilage Organism	Colony Count (CFU/mL)			
	No COS	100 mg/L COS	500 mg/L COS	1000 mg/L COS
Enterococcus	$(1.46 \pm 0.59) \times 10^8$	$(1.15 \pm 0.54) \times 10^8$	$(1.81 \pm 0.45) \times 10^4$	$(5.30 \pm 2.10) \times 10^2$
Bacillus cereus	$(2.39 \pm 1.42) \times 10^8$	$(6.45 \pm 2.12) \times 10^7$	$(1.61 \pm 0.93) \times 10^3$	$(1.99 \pm 0.30) \times 10^2$
Paenibacillus	$(1.71 \pm 1.03) \times 10^8$	$(6.16 \pm 2.58) \times 10^7$	$(8.47 \pm 3.12) \times 10^3$	$(1.73 \pm 0.65) \times 10^2$
Alkaliphilus	$(1.83 \pm 0.77) \times 10^7$	$(4.24 \pm 1.31) \times 10^6$	$(4.92 \pm 1.63) \times 10^2$	0
Pantoea sp.	$(2.20 \pm 1.25) \times 10^8$	$(1.10 \pm 0.20) \times 10^7$	$(1.85 \pm 0.51) \times 10^5$	$(9.33 \pm 6.66) \times 10^2$
Lactococcus	$(1.77 \pm 0.12) \times 10^7$	$(1.55 \pm 0.31) \times 10^7$	$(5.67 \pm 2.73) \times 10^2$	0
Aspergillus flavus	$(1.61 \pm 0.76) \times 10^8$	$(1.93 \pm 0.66) \times 10^7$	$(1.89 \pm 0.74) \times 10^3$	$(1.23 \pm 0.45) \times 10^2$
Aureobasidium pullulans	$(2.10 \pm 0.41) \times 10^6$	$(5.88 \pm 1.81) \times 10^5$	$(6.43 \pm 3.41) \times 10^3$	$(7.33 \pm 3.66) \times 10^1$
Penicillium	$(1.52 \pm 0.15) \times 10^7$	$(8.51 \pm 3.17) \times 10^6$	$(6.87 \pm 2.54) \times 10^4$	$(2.55 \pm 0.67) \times 10^3$

Values are expressed as mean ± SD.

3.2. Comparative Analysis of COS and SO_2 Antimicrobial Effects

SO_2 is a traditional food additive that has been permitted for use in wine. The total limited concentration of SO_2 in wine can vary, such as 200 mg/L or 300 mg/L, because laws restricting SO_2 use in wine vary among different countries. However, winemakers tend to supplement wine with as little SO_2 as possible owing to negative health effects on consumers with special sensitivity. To simulate the real environment of contamination during winemaking, nine types of wine spoilage organism were mixed together and inoculated into a pasteurized must. Free SO_2, rather than its bound form, can inhibit microbes, but the amount of free SO_2 is closely related to the temperature and pH of a must.

Therefore, the fermentation temperature was kept at 25 °C, and the pH values of all musts before fermentation were determined to be around 3.5. Finally, SO_2 (up to 200 mg/L) was added to investigate its antimicrobial effect. Surviving colonies were only reduced by 15% when supplemented with 50 mg/L SO_2 compared to that without additives. Therefore, 50 mg/L SO_2 was clearly insufficient for large-scale antimicrobial action. Increasing the SO_2 concentration to 100 or 200 mg/L significantly reduced the amount of surviving spoilage organisms, which finally reached less than 0.01%. The amount of surviving colonies with asupplementation of 500 mg/L COS was close to that achieved by adding 100 mg/L SO_2 (Table 2).

Table 2. Comparative analysis of antimicrobial effects on the mixture of different wine spoilage organisms among using 500 mg/L COS and 50, 100, and 200 mg/L SO_2 as additives.

Additive	Concentration (mg/L)	Colony Count (CFU/mL)
/	/	$(2.17 \pm 0.32) \times 10^7$
COS	500	$(1.73 \pm 0.57) \times 10^4$
SO_2	50	$(1.86 \pm 0.21) \times 10^7$
	100	$(1.34 \pm 0.31) \times 10^4$
	200	$(1.43 \pm 0.38) \times 10^3$

Values are expressed as mean ± SD.

Furthermore, comparative analysis of the antimicrobial effects of COS (500 mg/L) and SO_2 (100 mg/L) as individual additives on nine different types of wine spoilage organism was conducted. As shown in Table 3, 500 mg/L COS showed better antibacterial effects than 100 mg/L SO_2 on all the nine wine spoilage organisms, except *Enterococcus* and *Alkaliphilus*. For three types of fungi, COSsshowed greater antifungal effects than SO_2. This might be due to COS originating from chitin, which composes the fungal cell wall. Among the six types of bacteria, COS showed greater antibacterial effects on *Bacillus cereus*, *Paenibacillus*, *Pantoea* sp., and *Lactococcus* compared with SO_2, while SO_2 showed greater antibacterial effects on *Enterococcus* and *Alkaliphilus*. This implied that antimicrobial effects of COS or SO_2 varied among different microorganisms, which might be due to physicochemical characteristics of each microorganism type, including hydrophilicity and negative charge distributions on the cell surface [32]. Overall, 500 mg/L COS showed a greater antifungal effect than 100 mg/L SO_2, but both of the additives had a comparable antibacterial effect.

Table 3. Comparative analysis of antimicrobial effects on nine different wine spoilage organisms between using 500 mg/L COS and 100 mg/L SO_2 as additives.

Wine Spoilage Organisms	Colony Count (CFU/mL)		
	No COS	500 mg/L COS	100 mg/L SO_2
Enterococcus	$(1.81 \pm 0.39) \times 10^3$	$(6.60 \pm 2.05) \times 10^2$	$(1.6 \pm 0.47) \times 10^2$
Bacillus cereus	$(1.49 \pm 0.62) \times 10^3$	$(5.90 \pm 1.93) \times 10^2$	$(6.33 \pm 0.30) \times 10^2$
Paenibacillus	$(2.14 \pm 1.01) \times 10^3$	$(3.20 \pm 1.06) \times 10^2$	$(5.50 \pm 3.69) \times 10^2$
Alkaliphilus	$(1.94 \pm 0.37) \times 10^3$	$(1.92 \pm 0.74) \times 10^2$	$(5.20 \pm 2.28) \times 10^1$
Pantoea sp.	$(1.10 \pm 0.20) \times 10^2$	$(3.33 \pm 1.52) \times 10^1$	$(4.96 \pm 1.30) \times 10^1$
Lactococcus	$(1.77 \pm 0.52) \times 10^4$	$(1.83 \pm 0.09) \times 10^2$	$(9.03 \pm 0.22) \times 10^3$
Aspergillus flavus	$(1.39 \pm 0.16) \times 10^3$	$(6.56 \pm 2.81) \times 10^2$	$(9.96 \pm 2.25) \times 10^2$
Aureobasidium pullulans	$(7.10 \pm 3.11) \times 10^3$	$(2.10 \pm 0.71) \times 10^2$	$(2.33 \pm 0.45) \times 10^2$
Penicillium	$(1.63 \pm 0.45) \times 10^3$	$(2.26 \pm 0.59) \times 10^2$	$(8.13 \pm 0.15) \times 10^2$

Values are expressed as mean ± SD.

3.3. Estimating Antimicrobial Effectsof COS on Known Wine Spoilage Microorganisms

As COS showed greater antimicrobial effects than SO_2 on our own separated wine spoilage microorganisms, including six types of bacteria and three types of fungi, its antimicrobial effects onknown wine spoilage microorganisms were also evaluated. Among known wine spoilage

microorganisms [33], *Pediococcus damnosus*, *Acetobacter aceti*, *Lactobacillus plantarum*, and *Lactobacillus brevis* were selected for evaluation. As shown in Table 4, the colony counts of spoilage microorganisms grew during the first few days and then dropped rapidly owing to the increasing ethanol content. Neither SO_2 nor COS completely killed the microbes, but an inhibited microbe growth was observed. As ethanol resistance was different among these four wine spoilage microorganisms, their survival times during fermentation were different. The growth curve of each type of bacteria with 500 mg/L COS was always slightly below those with 100 mg/L SO_2 and far below those with no additive, as determined by the colony count from each time point (Table 4). Therefore, 500 mg/L COS showed stronger antibacterial effects comparable to 100 mg/L SO_2.

Table 4. The colony counts of four known wine spoilage organisms at each time point (24, 48, 72, 96, and 120h) after using 500 mg/L COS or 100 mg/L SO_2 as an additive.

Colony Count (CFU/mL)		24 h	48 h	72 h	96 h	120 h
Lactobacillus plantarum	/	$(1.54 \pm 0.52) \times 10^6$	$(2.74 \pm 0.58) \times 10^7$	0	–	–
	COS	$(1.77 \pm 0.41) \times 10^5$	$(5.08 \pm 1.50) \times 10^5$	0	–	–
	SO_2	$(1.98 \pm 0.23) \times 10^4$	$(5.93 \pm 2.23) \times 10^5$	0	–	–
Acetobacter aceti	/	$(5.83 \pm 2.67) \times 10^6$	$(9.10 \pm 1.73) \times 10^7$	$(4.33 \pm 1.96) \times 10^8$	$(1.60 \pm 0.16) \times 10^2$	0
	COS	$(1.95 \pm 0.18) \times 10^4$	$(1.24 \pm 0.10) \times 10^5$	$(5.57 \pm 0.97) \times 10^6$	$(6.83 \pm 1.76) \times 10^1$	0
	SO_2	$(1.61 \pm 0.45) \times 10^5$	$(3.13 \pm 1.35) \times 10^6$	$(2.69 \pm 0.45) \times 10^7$	$(6.96 \pm 1.91) \times 10^1$	0
Pediococcus damnosus	/	$(1.83 \pm 0.23) \times 10^6$	$(9.23 \pm 3.95) \times 10^6$	$(2.85 \pm 0.24) \times 10^7$	$(7.70 \pm 1.25) \times 10^1$	0
	COS	$(6.47 \pm 3.88) \times 10^4$	$(2.23 \pm 0.51) \times 10^5$	$(7.50 \pm 1.27) \times 10^5$	$(1.80 \pm 0.43) \times 10^1$	0
	SO_2	$(1.16 \pm 0.47) \times 10^4$	$(2.72 \pm 0.62) \times 10^5$	$(9.00 \pm 1.92) \times 10^5$	$(4.23 \pm 1.02) \times 10^1$	0
Lactobacillus brevis	/	$(9.73 \pm 1.19) \times 10^5$	$(9.10 \pm 2.46) \times 10^6$	$(1.04 \pm 0.59) \times 10^2$	0	–
	COS	$(2.75 \pm 0.37) \times 10^4$	$(1.78 \pm 0.58) \times 10^5$	$(2.33 \pm 0.77) \times 10^1$	0	–
	SO_2	$(5.37 \pm 2.94) \times 10^3$	$(2.43 \pm 0.59) \times 10^5$	$(2.93 \pm 1.38) \times 10^1$	0	–

Values are expressed as mean ± SD.

3.4. Effects of COS on Saccharomyces Cerevisiae and PichiaPastoris Cell Growths

To investigate the effects of COS on the cell growths of *Saccharomyces cerevisiae* and *Pichiapastoris*, a pasteurized must with 500 mg/L COS as an additive was inoculated with only *Saccharomyces cerevisiae* or *Pichiapastoris*, without any wine spoilage organism. A pasteurized must without any additive and inoculated with only *Saccharomyces cerevisiae* or *Pichiapastoris* was used as a control group. The bacterial concentration was evaluated using the OD_{600} value. The logarithmic phase of the cell growth of *Saccharomyces cerevisiae* was slightly extended, when 500 mg/L COS was added, because bacterial growth was slightly slowed down. When the stationary phase was reached, the bacterial concentrations (OD_{600} values) with or without COS were not significantly different. However, the growth of *Pichiapastoris*, a type of wild yeast that can cause wine spoilage [1], was significantly inhibited by 500 mg/L COS (Figure 2). Therefore, 500 mg/L COS supplemented into the must inhibited *Pichiapastoris* cell growth but did not influence *Saccharomyces cerevisiae* cell growth. Therefore, using 500 mg/L COS as an additive during winemaking would not affect the fermentation process.

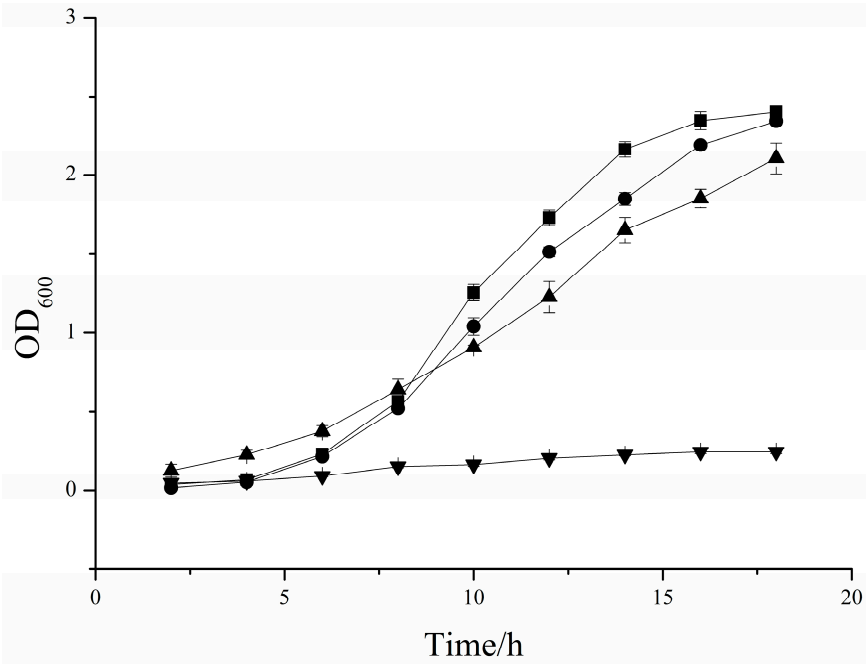

Figure 2. Effects of 500 mg/L COS on the cell growths of *Saccharomyces cerevisiae* and *Pichiapastoris* during winemaking that were determined by using the optical density (OD) valuesat 600 nm (OD_{600}). ■, *Saccharomyces cerevisiae* withoutCOS; ●, *Saccharomyces cerevisiae* withCOS; ▲, *Pichiapastoris* withoutCOS; ▼, *Pichiapastoris* withCOS.

As summarized in Table 5, compared with previously reported multiple additions and physical methods, COS showed a more universal antimicrobial effect without any adverse effects. Using 500 mg/L COS as an additive during winemaking did not influence *Saccharomyces cerevisiae* cell growth, meaning that COS did not influence the fermentation process. Furthermore, the radical scavenging activity of wine could be increased by adding COS, as confirmed by analysis of the DPPH and hydroxyl radical scavenging activities (data not shown). Therefore, COS shows potential for applications in the winemaking industry.

Table 5. Experimentally analyzed parameters when studying SO_2 replacement in wine. (referred to [34] with minor modification).

Replacement	Experimental Conditions	Oenological Parameter	Studied Property					
			Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial
Addition	Dimethyl dicarbonate [8]	Supplemented concentration of DMDC containing 25, 50 and 100 mg/L; Comparison between added SO2 with different concentrations in wine before bottling.						
	Bacteriocin [9]			MRS agar for lactic acid bacteria;MLOagar forO. *oeni*; Mannitol agar plates, 5 g/L yeast extract, 3 g/L peptone, and 15 g/L agar for acetic acid bacteria; MIC, MBC, MIC_{50}, MIC_{90}, MBC_{50}, and MBC_{90}			
	Phenolic compounds [10]	Control group, 160 mg/L SO_2; Winery-scale trial		O. *oeni*, *L. hilgardii*, and *P. pentosaceus*; Cultures prepared by adding ethanol; IC_{50}, MIC, and MBC; OD_{600}; Electron microscopy	Dipyridyl method;2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid (ABTS) method	Major alcohols, esters, and acids; Esters, alcohols, terpenes, C13-norisoprenoids, acids, volatile phenols, lactones, furanic compounds, and vanillin compounds		Tasting at the end of alcoholic fermentation
	Lysozyme [12]	SO_2 addition 120 mg/L; No sulphur addition in control tests; Low=SO_2-production yeast used; Studied at alcoholic fermentation followed by malolactic fermentation one month later; Must at crushing, wine during and after alcoholic and malolactic fermentation	pH, density, absorbance at 420 nm, total polyphenol index, alcoholic strength, and dry extract	Volatile acids; WLN agar medium to enumerate yeast and MRS supplemented with 20% apple juice to enumerate lactic acid bacteria; IC_{50}; Viable microorganism counts were obtained by the number of CFU per mL	Oxygen radical absorbance capacity (ORAC)	Major alcohols, esters, and acids.		Tasting one week after bottling and after two-month storage; The Expert panel conducted a descriptive analysis of each wine; Triangle difference tests were performed; Panellists were given an option to comment on differences observed in the wines.

Table 5. *Cont.*

Replacement	Experimental Conditions	Oenological Parameter	Studied Property					
			Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial
	COS	The optimized concentration is 500mg/L; The compared additive is 100mg/L SO_2.		-CFUs *in Enterococcus, Bacillus cereus, Paenibacillus, Alkaliphilus, Pantoea* sp., *Lactococcus, Aspergillus flavus, Aureobasidium pullulans, Penicillium*; CFUsin *Lactobacillus plantarum, Acetobacter aceti, Pediococcus*; *damnosus, Lactobacillus brevis*; Growth curves of *Saccharomyces cerevisiae* and *Pichia pastoris*				
Physical methods	Pulsed electric fields [14]	SO_2 addition 40 mg/L; No sulphur addition in control tests; 12 months of ageing after bottling	pH, total acidity, volatile acidity, sugars, and ethanol; -Colour intensity, total anthocyanins, and total polyphenol index; CIELab coordinates;	*Dekkera anomala, D. bruxellensis, L. hilgardii*, and *Lactobacillus plantarum*; Response variable (S) in experimental designs	Along the time of storage, the wines showed different evolutionS of total phenolics and antioxidant activity values; Folin–Ciocalteu method	Alcohols, esters, and acids.	Color intensity, anthocyanin content, and total polyphenol index	Tasting nine months after bottling
	Ultraviolet [13]	SO_2 addition 50 mg/L; Fresh and frozen must; Produced wine.	NIR multiparametric analysis; Tartaric acid, alcoholic degree, and volatile acidity; Absorbance spectrum	Density	Matrix color influenced the antioxidant analysis.			

Table 5. *Cont.*

Replacement	Experimental Conditions	Oenological Parameter	Studied Property					
			Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial	Antimicrobial
	High hydrostatic pressure [15]			Growth of *D. bruxellensis* using a selective/differential medium; Analysis of 4-ethylphenol by GC EMS	Anthocyanins and proanthocianins by HPLC-DAD-TQD	Analysis of volatile compounds by GCFID		
	Ultrasound [14]	Laboratory scale	pH, total acidity, volatile acidity, sugar, and ethanol					

4. Conclusions

COS with an optimal concentration of 500 mg/L exerted antimicrobial activity against nine of our own separated wine spoilage organisms and five types of known wine spoilage organism. The antimicrobial effect of 500 mg/L COS was comparable to that of 100 mg/L SO_2. Furthermore, using 500 mg/L COS as an additive during winemaking did notinfluence *Saccharomyces cerevisiae* cell growth. Therefore, COS is a potential replacement for SO_2 in winemaking.

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