Research Note

The Potential Application of Vanillin in Preventing Yeast Spoilage of Soft Drinks and Fruit Juices

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ABSTRACT

The preservative effect of vanillin, the major constituent of vanilla beans, was studied in an apple juice and peach-flavored soft drink. Vanillin activity was tested against Saccharomyces cerevisiae and Candida parapsilosis at an 8-week storage period. Initial results in laboratory media indicated minimum inhibitory concentration values of 17 and 9 mM vanillin for the two yeast strains. Concentrations of 20 and 10 mM vanillin, respectively, were required to achieve complete inhibition of both yeast strains inoculated at a level of \(1 \times 10^4\) CFU/ml in the apple juice and peach-flavored soft drink over the 8-week storage at 25°C. These effective levels were reduced to 5 and 1 mM, when the storage temperature was reduced to 8°C. A biocidal effect against both yeasts was observed within 96 h to 8 weeks, with vanillin concentrations of 5 to 40 mM depending on the beverage and the storage temperatures used. The increased activity of vanillin in the peach-flavored soft drink (pH 3.1) in comparison to the apple juice (pH 3.5) is probably a result of the lower intrinsic pH of the former; however, variation in vitamin and mineral levels or the presence of other phenolic compounds between the two drinks might also have contributed to the observed differences. Furthermore, the increased activity at the lower temperature could be linked to the combination of the increased membrane fluidity and the membrane-perturbing action of vanillin. We conclude that vanillin has the potential to preserve fruit juices and soft drinks that are low in both lipid and protein content against \(S. \text{cerevisiae}\) and \(C. \text{parapsilosis}\).

Spoilage of food products by microorganisms results in the waste of a valuable resource and incurs large financial losses for the food industry that will inevitably affect the consumer (16, 23). Fruit juices, fruit-based beverages, and soft drinks are targets for spoilage by yeasts, molds, and acid-tolerant bacteria. The adverse environment inflicted by the low pH, low oxygen levels, low concentration of nitrogen-containing compounds, and high sugar content of these drinks prevents the growth of most microorganisms (11, 27). However, these hurdles do not inhibit the growth of many spoilage yeasts. A study of the yeast flora of frozen fruit juice concentrates reported the isolation of 12 genera and 21 species of yeast (10). Saccharomyces cerevisiae was the most frequently isolated yeast species (24.7%) followed by Candida stellata (22.1%) and Zygosaccharomyces rouxii (14.3%). Five other Candida strains, including Candida parapsilosis, were also isolated.

Typically the shelf life of beverages can be extended by pasteurization, the addition of chemical preservatives such as acids or sulfur dioxide, but most commonly by refrigeration (1, 11). There is pressure from consumers for food manufacturers to produce food that is minimally processed and free from chemical preservatives (1, 4, 13). The application of natural compounds with antimicrobial properties to food products might provide an alternative to the “chemical” preservatives currently employed (3, 9, 13, 14). Spices, herbs, and plant essential oils added to foods primarily as flavoring agents have been shown to possess a broad range of antimicrobial activity (2, 8, 17, 28).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) occurs naturally as the major constituent of vanilla beans. Vanillin has generally recognized as safe (GRAS) status and is added as a flavoring to foods such as ice cream, confectionery, chocolate, and liquors (15, 22). It is also an effective inhibitor of yeast and mold growth in laboratory media, fruit-based agar systems, and fruit purees (5, 6, 7, 12, 18, 21). The growth of \(S. \text{cerevisiae}\), Zygosaccharomyces bailii, Z. rouxii and Debaryomyces hansenii was inhibited for 40 days in both laboratory media and apple puree when vanillin was added at a concentration of approximately 13 mM (6). Vanillin was found to be less effective when added to banana puree; a concentration of approximately 20 mM was insufficient to inhibit the growth of \(Z. \text{bailii}\); the authors attributed the lack of antimicrobial activity to the high lipid/protein levels found in bananas. Such interactions reduce the quantity of vanillin available to act in an antimicrobial context in any given system. Certain fruit juices and soft drinks contain only trace amounts of protein and lipids; this along with their “sweet” characteristics could complement both the antimicrobial activity and sweet aroma attributed

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to vanillin if this compound was to be added as a natural preservative.

The present study determined the antimicrobial activity of vanillin when applied to an apple juice and peach-flavored soft drink inoculated with *S. cerevisiae* or *C. parapsilosis* at two different incubation temperatures of 25 and 8°C.

**MATERIALS AND METHODS**

**Yeast strains and culture conditions.** *S. cerevisiae* (NCYC 956) was obtained from the National Collection of Yeast Cultures, Norwich, UK. *C. parapsilosis* was a gift from Unilever R&D, Bedford, UK. Yeast cells were maintained at 2°C on yeast extract peptone dextrose (YEPD) agar containing 20 g glucose (BDH, Poole, Dorset, UK), 20 g bacteriological peptone (Becton Dickinson, Oxford, UK), 10 g yeast extract (Difco, Oxford, UK), and 15 g agar (Difco) per liter. Yeasts were grown overnight (18 h) in YEPD broth (pH 4.0) at 25°C with continuous agitation.

**Vanillin preparation.** A stock solution of 2.5 M vanillin (Sigma, Poole, Dorset, UK) was prepared in 99.7 to 100% (vol/vol) ethanol. This solution was stored at −20°C in the dark until required.

**Susceptibility testing.** Susceptibility of the yeast cultures to vanillin is expressed as the MIC. Overnight cultures of *S. cerevisiae* and *C. parapsilosis* were subcultured (1% vol/vol) into fresh YEPD (pH 4.0) and transferred to a sterile 100-well microtiter plate. Vanillin was then added to give final concentrations ranging from 5 to 20 mM in triplicate in a final volume of 400 μl per well. Cultures grown in the absence or presence of 2% (vol/vol) ethanol acted as controls. Growth was measured by turbidity (OD$_{600}$) with a Labsystems Bioscreen C (Thermo Life Sciences, Basingstoke, Hampshire, UK) for 96 h at 25°C without agitation.

**Beverage preparation.** Both beverages used in our experiments were purchased locally and stored at 4°C according to the manufacturers’ instructions. The pure apple juice contained 11.5 g sugars, 0.1 g protein, and trace amounts of fat per 100 ml and had undergone pasteurization during its production. The peach-flavored soft drink contained 8 to 10 g sugars, peach juice, and peach flavoring per 100 ml and had been pasteurized during its production. The pH of the beverages was confirmed in the laboratory to be pH 3.5 and pH 3.1 for the apple and peach drinks, respectively. Neither product contained any preservatives.
FIGURE 3. The growth of *S. cerevisiae* in the presence of 1 mM (■), 5 mM (□), 10 mM (○), 20 mM (●), or 40 mM (▲) vanillin in peach-flavored soft drink at (A) 25°C and (B) 8°C. Growth in the presence of 1.6% (vol/vol) ethanol (∆) acted as the control. Values represent the mean of duplicate samples surface plated in triplicate.

FIGURE 4. The growth of *C. parapsilosis* in the presence of 1 mM (■), 5 mM (□), 10 mM (○), 20 mM (●), or 40 mM (▲) vanillin in peach-flavored soft drink at (A) 25°C and (B) 8°C. Growth in the presence of 1.6% (vol/vol) ethanol (∆) acted as the control. Values represent the mean of duplicate samples surface plated in triplicate.

The beverages were aseptically dispensed (25 ml) into sterile 30-ml screw-capped bottles. These conditions simulated those of a sealed, bottled beverage. Both products were initially checked for absence of contaminants by surface plating onto YEPD agar and inoculation into YEPD broth followed by incubation at 25°C for 72 h. Contaminants were not detected in either drink product.

**Inhibition of cell viability.** Overnight yeast cultures were harvested by centrifugation at 2°C (4,400 × g for 10 min) and resuspended in quarter strength Ringers solution (pH 7.0). The cultures were diluted in the same solution until an OD_{600} of 0.6 was attained. The beverage samples containing different levels of vanillin were then inoculated in duplicate to give an initial viable cell count of approximately 1.0 × 10^4 CFU/ml. Samples (20 μl) were taken immediately, and the decimal dilutions made in quarter strength Ringers solution were surface plated (in triplicate) onto YEPD agar. The viable cell counts were enumerated after incubation at 25°C for 48 to 72 h. Beverage samples containing 1.6% (vol/vol) ethanol acted as the controls. The inoculated sample sets were incubated at either 8 or 25°C. Further samples were taken at intervals over an 8-week period for enumeration of viable counts.

**RESULTS AND DISCUSSION**

**Antimicrobial activity of vanillin in laboratory media.** Results showed that *C. parapsilosis* was nearly twice as susceptible as *S. cerevisiae* with MICs established at 9 and 17 mM, respectively, after incubation at 25°C for 96 h. In a previous study (6), it was reported that the addition of approximately 13.2 mM vanillin to laboratory media with water activity of 0.99 was sufficient to inhibit the growth of *S. cerevisiae* for 40 days. The differences in inoculum size (1.0 × 10^7 CFU/ml compared to approximately 1.0 × 10^4 CFU/ml used here), the growth media used, or the specific *S. cerevisiae* strains could have accounted for the higher MIC reported here. Increasing the concentration of vanillin resulted in the extension of the lag phase of growth and the reduction of cell density (data not shown). Our previous study (12) with laboratory-based media indicated that at MIC, the activity of vanillin against the spoilage yeasts was biostatic rather than biocidal.

**Antimicrobial activity of vanillin in apple juice.** Sub-MIC levels of vanillin had little or no effect on con-
trolling the growth of *S. cerevisiae* in apple juice at 25°C (Fig. 1A). The addition of 20 mM vanillin inhibited growth for 96 h with a slight reduction in the viable counts. *S. cerevisiae* cells were no longer detected in the juice after only 96 h or 1 week in the presence of 40 and 20 mM vanillin, respectively. Lowering the storage temperature to 8°C significantly improved the effectiveness of vanillin against this yeast culture (Fig. 1B). Lower temperature extended the lag phase and decreased growth rates of all culture samples compared to those grown at 25°C. The presence of 5 mM vanillin was sufficient to inhibit growth for the duration of the study, with a parallel reduction in viable cell counts to 2.5 × 10^2 CFU/ml. Furthermore, the additions of 10, 20, and 40 mM vanillin resulted in the complete loss of viability within 8, 4, and 3 weeks, respectively. These results are consistent with those of a previous study that reported that the activity of vanillin against a number of *Aspergillus* strains could be increased by lowering the incubation temperature from 25 to 15°C (19).

Additions of either 5 or 10 mM vanillin extended the lag phase and decreased the growth rate of *C. parapsilosis* but were unable to prevent the spoilage of the juice at 25°C (Fig. 2B). Viable cells were not detected after 1, 3 and 6 weeks when the yeast was grown in the presence of 40, 20, or 10 mM vanillin. Additions of 5 or 10 mM vanillin again resulted in the complete loss of viability within 2 weeks and 96 h, respectively. The antimicrobial activity of vanillin was again enhanced by lowering the storage temperature to 8°C (Fig. 2B). Viable cells were not detected after 1, 3 and 6 weeks when the yeast was grown in the presence of 40, 20, or 10 mM vanillin.

Because of their hydrophobic nature, phenolic compounds preferentially partition into the cytoplasmic membrane, disrupting its integrity via interactions with the lipids in the bilayer, membrane-embedded proteins, or both (25, 26, 29). Work conducted in our laboratory using several bacterial strains indicated that vanillin is also a membrane-active compound (unpublished data). The biocidal activity of vanillin observed here could result from the intrinsically low pH of the apple juice (pH 3.5). In a previous study (20), vanillin and pH synergistic effects were observed in the inhibition of a number of *Aspergillus* strains when the pH was reduced from 4.0 to 3.0. The loss of pH homeostasis could be critical at lower external pH because of the probability that key enzyme functions would be detrimentally affected, thereby contributing to the observed inhibition that ultimately results in cell death. At lower temperatures, the cell membrane becomes more fluid (24), which, combined with the membrane-perturbing action of vanillin, could account for the greater effectiveness of vanillin at 8°C.

Antimicrobial activity of vanillin in peach-flavored soft drink. The peach-flavored soft drink proved to be a harsher environment for yeast growth. In cultures of *S. cerevisiae* without vanillin, the viable cell count increased from about 1.2 × 10^4 to about 1.3 × 10^6 CFU/ml within 96 h at 25°C (Fig. 3A). The growth of this yeast was inhibited by the addition of 5 mM vanillin for a 2-week period. Additions of 10 or 20 mM vanillin resulted in the complete loss of viability after 2 or 1 week, respectively, whereas at 40 mM, vanillin viability was lost after only 96 h. At 8°C, even 1 mM vanillin was sufficient to inhibit *S. cerevisiae*, although no decrease in viable cell counts was observed (Fig. 3B).

Additions of 1 or 5 mM vanillin failed to completely inhibit the growth of *C. parapsilosis* in the drink at 25°C (Fig. 4A). Concentrations of 10 mM vanillin or greater were sufficient to inhibit cell viability after a period of time (96 h to 8 weeks), which was dependent on vanillin concentration. Reducing the storage temperature to 8°C resulted in the arrest of growth in the control cultures of *C. parapsilosis*, as well as in cultures containing up to 5 mM vanillin, but without any loss of viability (Fig. 4B). The addition of higher levels of vanillin again led to the complete loss of viability.

The reduction in cell density in control cultures of both yeast species and the enhancement of vanillin activity in the peach-flavored soft drink compared to those reported in the apple juice can be linked to the intrinsic properties of the former. Although differences in pH of the two drinks is likely to be the dominant factor in the observed differences in vanillin inhibition of the yeasts, levels of essential nutrients such as vitamins, minerals, and nitrogen-containing compounds or the presence of other phenolic compounds could also contribute to the sensitivity of the yeast to vanillin.

**CONCLUSION**

Concentrations of 20 and 10 mM vanillin were required to achieve the complete inhibition of both yeasts investigated in the apple juice and peach-flavored soft drink over the 8-week storage period at 25°C. These effective levels could be reduced to 5 and 1 mM when the storage temperature was reduced to 8°C, a temperature equivalent to open chiller displays and upper refrigerator temperatures (1, 24). Vanillin is currently added as a flavoring component to bakery products and beverages at levels between about 1.3 to 12.5 mM (15). The vanillin levels required to inhibit growth reported here at the lower temperature compare favorably for practical use in fruit juices and soft drinks that are low in both lipid and protein content, which are thought to interfere with the antimicrobial activity of vanillin (6, 18). Whether the levels required to inhibit growth would be acceptable for the organoleptic qualities of the fruit juice or peach-flavored soft drink is an important consideration and would need to be investigated further.

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**REFERENCES**


