A Research Note
Viability of Byssochlamys nivea in Apple Sauce Containing Sorbate, Benzoate and Sulfur Dioxide and Packaged Under Various Oxygen Levels

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ABSTRACT

The effects of potassium sorbate (50 and 100 ppm), sodium benzoate (200 and 400 ppm) and SO₂ (25 and 50 ppm) on growth and patulin production by Byssochlamys nivea in apple sauce packaged under various levels of oxygen were determined. A low level (1.4-2.3%) of oxygen in the headspace of sealed pouches protected B. nivea against loss of viability over a 13-month storage period at 21°C. No increase in population was observed in inoculated apple sauce with headspace oxygen contents of up to 9.5-9.7%. Sulfur dioxide was the most lethal preservative tested, 50 ppm causing complete inactivation within 4-months. Patulin was not detected in any of the test samples.

Spoilage of processed fruits and fruit products by Byssochlamys fulva and B. nivea has been recognized for over a half century (1,3). Ascospores of this mold are capable of withstanding thermal processing treatments given to many fruit products, and thus represent a potential problem to the preservation of products during subsequent storage.

Byssochlamys spp. are capable of growing under reduced oxygen tension (2,4), and may also produce toxic metabolites such as patulin, byssolchamalic acid and byssotoxin A, depending upon the strain and conditions of culture.

Little is known about the influence of traditional antifungal agents on growth and toxin production by B. nivea under low oxygen tension. Experiments were therefore designed to determine the effects of potassium sorbate, sodium benzoate and SO₂ on behavior of B. nivea in apple sauce packaged under various oxygen tensions.

MATERIALS AND METHODS

Preparation of apple sauce

Rome Beauty variety apples were purchased from the Georgia State Farmers’ Market, Forest Park. Apples were thoroughly washed with cold water and weighed so that the yield of apple sauce could be determined. The apples were quartered by hand, placed into a Rietz Thermascrrew (Model TL-9-K2204, Rietz Manufacturing Co., Santa Rosa, CA), and subjected to 95-100°C for approximately 8 min. This time/temperature relationship was adequate to soften the apple tissue and loosen the skins. The heated apples were immediately passed through a pulper (Ailing Lander Co., Sodus, NY) fitted with a 0.002-in. mesh cone. The discarded pulp, skins, seeds and stems were collected and passed through the pulper once again. The apple sauce was collected, passed through a steam kettle and heated to 94°C (200°F) to destroy most of the microflora that may have been present on the apples before processing.

The hot apple sauce was deposited in No. 10 cans and sealed using a Dixie Sealer (Dixie Canner Equipment, Athens, GA). Each can contained 2,750 g of apple sauce (pH 3.7), leaving approximately 4 cm of headspace. Cans were inverted, placed in a cooling chamber, and sprayed with cold water for 1 1/2 h. The cans were allowed to air dry 4 h before storing at 12°C until used for this study.

Culture and inoculum

Byssochlamys nivea NRRL-2615 was maintained at 4°C on potato dextrose agar (PDA, pH 5.5). Cultures (8-10 d old) grown at 30°C on PDA were harvested by flooding plates with sterile 0.1 M potassium phosphate buffer (pH 7.0) containing 0.01% Tween 80. Conidiospores were suspended in buffer by gently rubbing a sterile glass rod over the surface of the mycelial mat. The suspension was filtered through sterile glass wool and diluted with buffer to give an absorbance of 1.18 on a Bausch and Lomb Spectronic 20 Spectrophotometer at a setting of 620 nm. The diluted suspension gave a viable population of 2.0 - 2.5 x 10⁷ colony forming unit (CFU)/ml. All inocula were prepared just before inoculation of apple sauce.

Preservatives

Stock solutions of potassium sorbate (Monsanto Company, St. Louis, MO), sodium benzoate (Pfizer Inc., New York, NY) and sodium metabisulfite (Mallinckrodt Chemical Works, St. Louis, MO) were prepared in deionized water and filter-sterilized. Appropriate amounts of solutions were added to apple sauce to give 50 and 100 ppm of potassium sorbate, 200 and 400 ppm of sodium benzoate and 25 and 50 ppm of SO₂.

The volume of solution added to achieve desired levels of preservative did not exceed 0.25% of the volume of apple sauce.

Packaging

Approximately 100 g of the preservative-supplemented apple sauce was placed in flexible can pouches (Reynolds Metal Co., Richmond, VA). Pouches (18.5 x 12.5 cm) consisted of a three-layer laminated polyester/aluminum foil/polyethylene. Pouches were placed in an evacuation chamber (Multivac Chamber Model AG 500, Multivac Co., West Germany) and a vacuum was drawn to achieve headspace oxygen levels

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of 1.4 - 2.3%, 4.5 - 5.1% and 9.5 - 9.7%. When the desired vacuum was reached, the chamber was flushed with nitrogen and the pouch was heat-sealed. Pouches were stored at 21°C for periods up to 13 mo.

**Enumeration of B. nivea**

Pouches were opened after various periods of storage and the apple sauce was visually inspected for surface mold growth. Samples not showing growth were analyzed for viable population of B. nivea: 20g of sauce were combined with 80 ml of sterile 0.1 M potassium phosphate buffer containing 0.01% Tween 80 (pH 7.0) and homogenized for 1 min using a Colworth Stomacher. Portions (0.1 ml) of homogenate were plated on plate count agar (pH 6.8) containing 100 ppm each of chloramphenicol and chlortetracycline-HCl. Colonies of B. nivea were counted after 5 d of incubation at 21°C.

**Analysis for patulin**

During the course of the study, approximately 6% of the samples were found to have mold(s) growing on the surface when pouches were opened. These samples were analyzed for patulin content. Apple sauce and mycelial mat were homogenized at speed 3.5 for 1 min using a Polyton homogenizer (Kinematica GmbH, Luzern, Switzerland). Ten grams of homogenate were combined with 20 ml of ethyl acetate and vigorously mixed. After separation of sauce and solvent layers, the ethyl acetate extract was removed and the extraction procedure was repeated. Combined extracts were dried over approximately 20 g of anhydrous NaSO 4 for 20 min and evaporated to approximately 25 ml using a rotary flash evaporator.

Thin-layer chromatography was carried out on 20 x 20 cm, 250 μK5 silica gel plates (Whatman Chemical Separation Division, Clifton, NJ). The plates were scored to produce 1-cm columns, 15 cm in height. Samples were spotted in 1-μl portions of sauce extracts, 1 cm from the bottom of the plate. The plates were developed in an equilibrated glass tank with toluene:ethyl acetate:90% formic acid (5:4:1, v/v/v) and allowed to air dry at room temperature. Plates were lightly sprayed with 4% phenylhydrazine-hydrochloride and heated at 110°C for 2-3 min. Patulin appears as a yellow spot (R f value of 0.60) under visible light.

Quantitation of patulin was made by measuring the intensity of spots using a photodensitometer model 520-A (Photovolt Corporation, New York, NY).

To determine the percentage recovery of patulin from apple sauce, an aqueous solution (pH 4.0) of patulin standard (5 mg/ml) was added to the sauce to give concentrations ranging from 50 to 700 μg/kg. The spiked apple sauce was allowed to stand overnight before analysis. Duplicate samples of sauce containing various known levels of patulin were analyzed and the percentage recovery was calculated.

**RESULTS AND DISCUSSION**

Data showing the effects of various levels of oxygen in headspace and preservatives in apple sauce on viability of B. nivea over a 13-month period of storage at 21°C are listed in Table 1. It should be noted that propagules of B. nivea consisted largely of conidiospores. The formation of ascospores was not observed in the young culture originally used as an inoculum. In no instance was there an increase in CFU/g of apple during storage. A low level of oxygen (4.6 - 5.1% and below) in headspace afforded protection against loss of viability over the 13-month storage period compared to the highest oxygen level (9.5 - 9.7%) tested.

Sulfur dioxide, at 50 ppm, was lethal to B. nivea. No viable cells were detected after 4 months of storage. After 13 months of storage, 200 and 400 ppm of sodium benzoate and 25 ppm of SO2 exerted approximately the same lethal effect on B. nivea, regardless of the oxygen level. Under the highest oxygen level tested (9.5 - 9.7%), 50 and 100 ppm of potassium sorbate caused a detrimental effect similar to that of 200 and 400 ppm of sodium.

**TABLE 1. Influence of low O2 tension and preservatives on the viability of B. nivea in apple sauce stored at 21°C for 4 and 13 months.**

<table>
<thead>
<tr>
<th>Headspace oxygen content (%)</th>
<th>Preservative</th>
<th>Type</th>
<th>Conc (ppm)</th>
<th>Colony forming units/ga</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 - 2.3</td>
<td>control</td>
<td>0</td>
<td>3000</td>
<td>3300</td>
</tr>
<tr>
<td></td>
<td>K sorbate</td>
<td>50</td>
<td>1900</td>
<td>1800</td>
</tr>
<tr>
<td></td>
<td>Na benzoate</td>
<td>200</td>
<td>400</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>200</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>SO2</td>
<td>25</td>
<td>2400</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.6 - 5.1</td>
<td>control</td>
<td>0</td>
<td>4000</td>
<td>4300</td>
</tr>
<tr>
<td></td>
<td>K sorbate</td>
<td>50</td>
<td>2300</td>
<td>1700</td>
</tr>
<tr>
<td></td>
<td>Na benzoate</td>
<td>200</td>
<td>1200</td>
<td>8</td>
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<td></td>
<td></td>
<td>400</td>
<td>700</td>
<td>21</td>
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<tr>
<td></td>
<td>SO2</td>
<td>25</td>
<td>3000</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.5 - 9.7</td>
<td>control</td>
<td>0</td>
<td>5600</td>
<td>180</td>
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<tr>
<td></td>
<td>K sorbate</td>
<td>50</td>
<td>1300</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Na benzoate</td>
<td>200</td>
<td>2400</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>2700</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>SO2</td>
<td>25</td>
<td>3500</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are averages of duplicate (4 months) and quadruplicate (13 months) determinations. Initial CFU per g of apple sauce was 24000.
benzoate and 25 ppm of \( \text{SO}_2 \); however, at lower oxygen levels, potassium sorbate was not as effective. All levels of preservatives resulted in a decrease in \( B. \text{nivea} \) compared to controls.

The concentrations of preservatives selected were based on previous observations showing that, on an equivalent weight basis, \( \text{SO}_2 \) was more inhibitory to growth of \( B. \text{nivea} \) in apple juice than was potassium sorbate, which in turn was more inhibitory than sodium benzoate (6). Although the latter studies were carried out under aerobic conditions, it appears that the inhibitory effect of \( \text{SO}_2 \) was highest, regardless of oxygen tension.

Patulin was not detected in samples of apple sauce analyzed throughout the course of the study. Molds observed growing on the surface of samples included \( B. \text{nivea} \) as well as other species. Also, there was no correlation between the appearance of mold and calculated level of preservative or oxygen, suggesting that growth occurred due to changes in oxygen tension resulting from defective seals in pouches.

Growth of \( \text{Byssochlamys} \) has been observed in cans and bottles of fruit with a limited amount of headspace (3). King et al. (2) reported that \( B. \text{fulva} \) was capable of growing in an atmosphere containing as little as 0.22% oxygen at a flow rate of 10 L/h. The mold was unable to grow on PDA, Czapek solution agar or grape juice under strictly anaerobic conditions. Orth (4) demonstrated that \( B. \text{nivea} \) produced patulin in apple juice in an environment containing 0.5 to 2% oxygen, and Rice (5) reported that cultures of \( B. \text{fulva} \) and \( B. \text{nivea} \) produced less patulin in grape juice packed in glass jars with headspaces of 2.5 and 1.3 cm than in jars with a headspace of 5.1 cm. He also reported that \( B. \text{nivea} \) lowered the oxygen level to approximately 0.5%, at which point the mold was unable to lower the oxygen concentration further. This low oxygen level inhibited further growth and patulin production.

In the present study, \( B. \text{nivea} \) was not observed to grow on apple sauce sealed in pouches initially containing as much as 9.5 - 9.7% oxygen. This level may have been reduced in the early stages of storage, however, due to adsorption of oxygen into the apple sauce which previously had been processed and sealed under conditions which reduced oxygen tension. Further analyses are needed to determine the oxygen level in headspace of pouches during and after equilibration with the apple sauce before the likelihood of growth and patulin production by \( B. \text{nivea} \) can be predicted.

**REFERENCES**