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Antimicrobial Effect of Butylated Hydroxyanisole and Butylated Hydroxytoluene on *Staphylococcus aureus* [1]

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ABSTRACT

The antimicrobial effect of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on three enterotoxigenic strains of *Staphylococcus aureus* in Brain Heart Infusion broth (BHI) was evaluated by turbidity measurements. Also, the interaction of these compounds with pH and NaCl on growth of *S. aureus* strain 100 was measured. Inhibition of *S. aureus* growth increased with an increase in the concentration of BHA and/or BHT. Complete inhibition of *S. aureus* growth occurred in BHI with 1.12 μmole of BHA/ml or 0.70 μmole of BHT/ml as well as with a combination of 0.25 μmole of both BHT and BHA/ml. Inhibition of *S. aureus* growth by BHA or BHT was substantial at pH 7.0 and with 2% NaCl. When 0.84 μmole or greater of BHA/ml and 0.47 μmole or greater of BHT/ml were added to BHI, growth of *S. aureus* 100 was inhibited to the extent that enterotoxin A could not be detected after 24 h of incubation.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used an antioxidants in foods and packaging materials. Recent reports show that these antioxidants also possess antimicrobial properties. Ward and Ward (*15*) found that a 1% concentration of BHT was slightly inhibitory to *Salmonella senftenberg*. Chang and Branen (*3*) reported that BHA had an antimicrobial effect against *Staphylococcus aureus*, enteropathogenic *Escherichia coli*, *Salmonella typhimurium* and *Aspergillus parasiticus*. *S. aureus* was the most sensitive of the bacteria tested and a 10^6 inoculum was totally inactivated by 150-200 ppm of BHA. Shih and Harris (*8*) reported that *S. aureus* and *E. coli* were inhibited in Trypticase Soy Broth (TSB) containing 100, 150, 200 and 400 ppm of BHA after 24 h of incubation. The amount of inhibition increased as the concentration of BHA increased. Recently VanTassell et al. (*14*) also reported that the growth of *S. aureus* in TSB was delayed for 36 h by 100 ppm of BHA while viable cell numbers decreased to less than 1 per ml within 3 h in the presence of 200 ppm BHA.

Microbes other than *S. aureus* are also affected by BHA and/or BHT. Surak (*12*) reported that both BHA and BHT were inhibitory to *Tetrahymena pyriformis*. Roback et al. (*6*) reported that *Vibrio parahaemolyticus* was inhibited in Trypticase Soy Broth containing 2.5% NaCl and 50 ppm BHA. Fung et al. (*4*) tested six toxigenic and six non-toxigenic strains of *Aspergillus flavus* to determine the effect of BHA and BHT on growth inhibition, spore formation, pigmentation and aflatoxin production. No inhibitory effect on growth and toxigenesis was observed with BHA (0.005-0.020 g per plate) while BHT (0.005-0.020 g per plate) gave no visible inhibitory effects.

The purpose of this study was to further define the antimicrobial properties of BHA and BHT toward *S. aureus*. The concentrations of BHA and BHT necessary to inhibit growth and enterotoxin production of *S. aureus* cultures and the influence of pH and NaCl on this inhibition were determined.

MATERIALS AND METHODS

Cultures

All enterotoxigenic strains of *S. aureus* (100, S-6, 361) used in this study were preserved in the dried form on porcelain beads using the method of Hunt et al. (*5*). Seed cultures were prepared by inoculating beads and incubating 18 h at 37 C. In the inhibition studies, BHI broth (100 ml) containing BHA and/or BHT was inoculated with a 1% inoculum from the seed cultures and incubated 24 h at 37 C.

BHA and BHT solutions and medium

The BHA and BHT used in this study was obtained from Eastman Kodak Company, Kingsport, Tennessee. One percent solutions of BHA and BHT were prepared by dissolving the antioxidants in 95% ethyl alcohol. Appropriate quantities of these solutions were added to BHI broth before sterilization at 121 C for 15 min. Preliminary experiments indicated that addition of these quantities of ethyl alcohol did not influence growth of *S. aureus*.

Growth measurement

Culture growth was measured turbidometrically using a Klett-Summerson coloriphotometer with a green filter (No. 54) of 520-580 nm wavelength. One hundred ml of BHI broth were added to a 250-ml Erlenmeyer flask with a Klett tube attached to the flask neck. The control flasks contained only BHI medium, whereas the experimental medium contained BHI plus BHA and/or BHT. The medium was inoculated, incubated without shaking and at specific intervals the flasks were agitated, tipped to fill the Klett tube and the turbidity was measured. Initial experiments indicated that maximum cell numbers and Klett readings were reached in 24 h in the control medium and in the medium containing BHA and/or BHT. Thus all experiments were terminated after 24 h and a Klett reading made at that time. Each trial was replicated and the two Klett readings were averaged. Percent inhibition was calculated by the following formula:

$$\%\text{inhibition} = \frac{(\text{Klett reading of control} - \text{Klett reading with BHA and/or BHT}) \times 100}{\text{Klett reading of control}}$$

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Enterotoxin production

After 24 h of incubation, 100 ml of the inoculated BHI broth was extracted for enterotoxin assay. The final extract volume was 0.2 ml. The extract was analyzed for enterotoxin by the microgel diffusion technique as described by Casman and Bennett (*2*). This technique has a sensitivity of 0.1 or 0.25 μg of enterotoxin per 100 g of substrate. In the work reported herein, no attempt was made to quantify the amount of enterotoxin present in the extract.

Effect of pH and NaCl

BHI broth containing BHA and/or BHT was adjusted before sterilization to pH 3, 4, 5, 6, 7, 8, or 9 using either 1.0 N NaOH or 1.0 N HCl. To determine the effect of NaCl, the BHI broth was prepared to contain 0, 2, 5, 10 or 15% NaCl and adjusted to pH 7 before sterilization.

RESULTS AND DISCUSSION

Three strains of *S. aureus* were tested against both BHA and BHT in Brain Heart Infusion (BHI) broth to determine if the antioxidants possessed antimicrobial properties (Table 1 and 2). Klett readings of the control cultures using *S. aureus* strain B were approximately 450 after 24 h of incubation at 37 C and the corresponding plate count was 3×10^{10}/ml. When 0.28 μmole and BHA/ml was added, the Klett readings were 400 after incubation and a plate count of 2×10^{9}/ml was obtained. A Klett reading of 350 was obtained and a plate count of 2×10^{8}/ml when 0.23 μmole of BHT/ml was added to the medium. When both BHA and BHT were added to the medium, Table 3, 0.28 μmole/ml and 0.23 μmole/ml, respectively, the initial Klett reading was 50 and after 60 h of incubation at 37 C, the Klett readings was still 50. The initial and concluding plate count was approximately 3×10^{7}/ml. Similar results were obtained with all three strains of *S. aureus* tested.

As the concentration of BHA or BHT was increased, the percent of inhibition also increased and total inhibition occurred with 1.12 μmoles of BHA/ml and 0.70 μmole of BHT/ml. In all the studies reported herein, BHT was more inhibitory than BHA. This observed difference in inhibitory action could be related to the greater lipolytic nature of BHT (*10*). Aaloto (*1*) also reported that as lipolytic nature of the phenols increases, the antimicrobial activity also increases. Similarly, Snipes et al. (*9*) reported that the ability of BHT to inactivate viruses was related to its lipolytic properties and its ability to associate with the lipid-containing membranes of the viruses. However, Fung et al. (*4*) reported BHA was more inhibitory to *A. flavus* than BHT. This is probably partially due to the lack of lipid material on the cell wall of *A. flavus*. These same workers also reported that BHA may be altering cell permeability, thus allowing leakage of macromolecules and may interact with cell membrane protein to cause disruption of membrane structure.

A combination of BHA and BHT had a greater inhibitory effect against *S. aureus* strain 100 than when either BHA or BHT was added alone (Table 3). Although 0.5 μmole of BHA/ml showed a 50% inhibition, a combination of 0.1 μmole of BHA/ml with 0.4 μmole of BHT/ml or 0.28 μmole of BHA/ml and 0.23 μmole BHT/ml resulted in 100% inhibition. The reason for this increased activity is not known; however, a similar synergistic effect has been noted in antioxidant activity when 0.1% BHA plus 0.1% BHT were used (*11*).

Detectable levels of enterotoxin A were produced by *S. aureus* strain 100 after 24 h in BHI broth alone, whereas enterotoxin was not detected after 24 h when 0.89 μmole or greater of BHA/ml, or 0.45 μmole or greater of BHT/ml, or a combination of 0.28 μmole BHA/ml plus 0.23 μmole BHT/ml were added.

Table 4 contains measurements of *S. aureus* strain 100 when incubated 24 h at 37 C in BHI medium adjusted to pH 3, 4, 5, 6, 7, 8 and 9 when 0.56 μmole BHA/ml or 0.45 μmole BHT/ml were added. Best growth occurred in the control medium (no BHA or BHT present) at pH 8 and 9 with gradual decrease down to pH 5 and then a slight increase in growth was noted at pH 3 and 4. When BHA or BHT were included in the medium, the least amount of inhibition occurred at the extreme pH values (3 and 9). Approximately 40 to 80% inhibition occurred when pH was between 5 and 8 with the 80% occurring with BHT at pH 7. Tompkin et al. (*13*) also reported

TABLE 1. *Inhibitory effect of BHA on three toxigenic strains of* Staphylococcus aureus *incubated 24 h at 37 C*[a].

BHA		% Inhibition		
ppm	mole/ml	100	S-6	361
0.0	0.00	0[b]	0	0
50	0.28	22	10	28
75	0.42	51	60	61
100	0.56	66	72	87
125	0.70	84	90	90
150	0.84	93	97	95
175	0.98	93	97	95
200	1.12	100	100	100

[a]BHI broth used at pH 7.0.
[b]Each value in the calculated mean of two replications.

TABLE 2. *Inhibitory effect of BHT on three toxigenic strains of* Staphylococcus aureus *incubated 24 h at 37 C*[a].

BHT		% Inhibition		
ppm	mole/ml	100	S-6	361
0.0	0.00	0[b]	0	0
50	0.23	42	23	39
75	0.35	65	56	76
100	0.45	97	93	94
125	0.58	97	93	94
150	0.70	100	100	100

[a]BHI broth used at pH 7.0.
[b]Each value is the calculated mean of two replications.

TABLE 3. *Inhibitory effect of the combination of BHA and BHT on the growth of* Staphylococcus aureus *strain 100 incubated 24 h at 37 C*[a].

mole/ml		
BHA	BHT	% Inhibition
0.0	0.0[b]	0
0.5	0.0	50
0.4	0.1	78
0.3	0.2	85
0.2	0.3	93
0.1	0.4	100
0.0	0.5	95
0.5	0.1	100
0.25	0.25	100

[a]BHI broth used at pH 7.0.
[b]Each value is the calculated mean of two replications.

TABLE 4. *Inhibitory effect of 0.56 μmole BGA/ml or 0.45 μmole BHT/ML in Brain Heart Infusion broth at different pH values on the growth of* Staphylococcus aureus *strain 100 incubated 24 h at 37 C*[a].

pH	Klett reading			Percent inhibition
	Control	BHI + BHA	BHI + BHT	
3	220	180		18
4	220	100		56
5	100	60		40
6	120	40		67
7	300	140		53
8	450	240		47
9	420	380		10
3	420		380	10
4	220		160	27
5	30		10	67
6	150		100	33
7	150		30	80
8	450		200	56
9	450		400	11

[a]Each value is the calculated mean of two replications.

maximum growth and enterotoxin A inhibition of *S. aureus* strain 100 at pH 7.0 in a medium containing sodium nitrite. The inhibition decreased as the medium was made more acidic or basic.

The data in Table 5 are the Klett readings and percent inhibition when *S. aureus* strain 100 was grown in BHI medium (pH 7.0) containing various concentrations of NaCl and BHA or BHT. As the NaCl concentration increased from 0 to 15%, without addition of BHA or BHT, the growth rate of *S. aureus* decreased, especially at the 10 and 15% levels of NaCl. The addition of 0.56 μmole BHA/ml, in the absence of NaCl, resulted in a 19% inhibition of growth. However, when 2% NaCl was included with the BHA, a 95% reduction in growth occurred and approximately the same reduction occurred when 5, 10 and 15% NaCl was included. When 0.45 μmole BHT/ml was added, an 87% reduction in growth occurred (Table 5). When NaCl was included, the percent inhibition ranged from 73 to 97%.

Addition of 2% or more NaCl enhanced the antimicrobial activity of BHA on *S. aureus* strain 100. The inhibitory effect of BHA, when in the presence of 2% NaCl, could be of importance in many food products since a 2% concentration of NaCl is commonly used (*7*). More research is necessary to determine the interactive effects of antioxidants, NaCl and pH on inhibition of *S. aureus*.

In conclusion, these studies indicate that BHA and BHT have marked antimicrobial effects on growth of three strains of *S. aureus* and enterotoxin production of one strain of *S. aureus*. A combination of BHA and BHT appeares to provide more effective inhibition than when the antioxidants are used alone. Both BHA and BHT are effective against *S. aureus* strain 100 in the range of pH and NaCl concentration encountered in food products. To ascertain the real effect of BHA and BHT in foods, commercial food items with and without BHA and BHT should be tested. Also, BHA and BHT can be added to food items known to be without these compounds and tested against *S. aureus*.

TABLE 5. *Inhibitory effect of sodium chloride and BHA or BHT in Brain Infusion broth on growth of* Staphylococcus aureus *strain 100 incubated 24 h at 37 C*[a].

% NaCl	μ mole/ml BHA	Klett reading	Percent inhibition due to:[b] NaCl	BHA	BHA + NaCl
0	0	540	0		
2	0	340	37		
5	0	440	19		
10	0	100	82		
15	0	60	89		
0	0.56	440		19	
2	0.56	20		94	95
5	0.56	40		91	91
10	0.56	60		40	86
15	0.56	20		67	95

% NaCl	μ mole/ml BHT	Klett reading	Percent inhibition due to:[b] NaCl	BHT	BHT + NaCl
0	0	600	0		
2	0	450	23		
5	0	400	33		
10	0	140	77		
15	0	40	97		
0	0.45	80		87	
2	0.45	60		87	90
5	0.45	160		60	73
10	0.45	40		71	73
15	0.45	20		50	97

[a]Each value is the calculated mean of two replications.
[b]% inhibition due to NaCl:

$$\%\,\text{inhibition} = \frac{\text{Klett reading of control} - \text{Klett reading with NaCl} \times 100}{\text{Klett reading of control}}$$

% inhibition due to BHA or BHT:

$$\%\,\text{inhibition} = \frac{\text{Klett reading of control} - \text{Klett reading with BHA or BHT} \times 100}{\text{Klett reading of control}}$$

% inhibition due to NaCl and BHA or BHT:

$$\%\,\text{inhibition} = \frac{\text{Klett reading of control} - \text{Klett reading with NaCl and BHA or BHT} \times 100}{\text{Klett reading of control}}$$

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