Inhibitory Effects of Butylated Hydroxyanisole on Growth of Molds and Yeasts at Different pH Conditions and Different NaCl Concentrations

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Abstract

The inhibitory activity of butylated hydroxyanizole (BHA) alone and at different pH (3.5 and 5.5) and different NaCl concentrations (2 and 4%) was screened on 5 molds and 3 yeasts. The conventional tube test was used to determine the minimum inhibitory concentration (MIC). BHA was effective against all of the microorganisms tested in the range of 20 to 320 µg/mL. The inhibition was pronounced at lowering pH and at NaCl concentrations. BHA was more effective in the presence of NaCl compared to acidic conditions. The MIC values of BHA was significantly lesser in media containing 4% and 2% NaC1 ($p < 0.05$). *Rhizopus arrhizus RAK* 220 was the most susceptible fungi to NaCl concentrations. In the presence of 2% NaCl concentration, the MIC value of BHA for *Rhizopus arrhizus RAK* 220 was reduced to ¼. The decrease in pH values from 7 to 5.5 and 3.5 had synergistic effect on the inhibitory activity of BHA on some microorganisms ($p < 0.05$). Among the tests, *Geotrichum candidum* UUFH 110 was the most susceptible fungi to reduce the pH values, as at pH 5.5 the MIC values of BHA reduced to ½ and at pH 3.5 reduced to one-sixth. In contrast, at different pH, the MIC values of BHA on *Candida albicans* CAL 512 not only not decreased but also increased. These studies show that the antifungal properties of BHA can be greatly influenced by environmental factors.

Keywords: Butylated hydroxyanizole (BHA), Mold, Yeast, pH value, NaCl concentration.

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Introduction

Antioxidants are very important not only for the prevention of food oxidation but also for the defense of living cells against oxidative damage.[1] Also they reduce harmful reactive free radicals and reactive oxygen species (ROS) in cells,[2] thereby preventing cancer and heart disease.[3] Phenolic antioxidants such as butylated hydroxyanisole (BHA), propyl p-hydroxybenzoate (propyl paraben (PP)), butylated hydroxytoluene (BHT) and propyl gallate (PG) are extensively used because of their excellent efficacy and low cost.[4-6] Because of their phenolic nature, several of these compounds used in foods primarily to prevent the auto-oxidation of lipids, they have also been shown to possess antimicrobial activity against a wide range of microorganisms.[4,7] From all four antioxidants,

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BHA and PP showed the strongest antimicrobial activity *in vitro*.[4]

Most evidence suggests that the inhibitory powers of BHA are, in part, due to disruption of the cytoplasmic membrane of cells. The compound may function as a membrane perturber by insertion into the lipid layer of the bacterial cell, causing disruption of the ordered state of the alkyl chains. Since the inhibitory effects of BHA are dependent primarily on lipid solubility and possibly on localization in lipid containing membranes. Also, differences in susceptibility reported for a variety of bacterial organisms may be related to the fatty acid composition of the species.[8]

Whereas most works focused on the antibacterial properties of BHA, little is known about the antifungal activity of these compounds. Therefore, the purpose of this investigation was to determine the antifungal activity of BHA alone and at different pH conditions and different NaCl concentrations.

Material and Methods

Chemicals

Butylated hydroxyanisole (BHA; 2, (3)-tert-butyl-4-hydroxyanisole), Sodium chloride (NaCl), Hydrochloric acid (Hcl), Sodium hydroxide (NaOH), Brain heart infusion broth (BHI) and Sabouraud dextrose agar (SDA) were obtained from Merck Chemical Co. (Merck, Darmstadt, Germany).

The stock solutions were prepared by dissolving the chemicals in appropriate solvent (either sterile distilled water or ethanol) and then sterilized by filtration through a 0.22 µm disposable sterile filter (Schleicher & schuell, Germany).[7,9]

Organisms

The test organisms were obtained from the culture collection of the Department of Microbiology, University of Tehran. These organisms included 5 molds (*Aspergillus niger* ASNG 1214, *Aspergillus flavus* ASNS 22, *Aspergillus fumigatus* ASN 2211, *Penicillium spp* PSIR 12, *Rhizopus arrhizus* RAK 220) and 3 yeasts (*Candida albicans* CAL 512, *Rhodotorula rubra* RRN 1214 and *Geotrichum candidum* UUFH 110).

Minimum Inhibitory Concentration (MIC)

Effect of BHA on the tested molds and yeasts

Minimum inhibitory concentration (MIC) values were examined for the microorganisms that were determined to be sensitive to BHA alone and at different pH conditions and different NaCl concentrations on the conventional tube test.[9,10] Firstly, BHA was dissolved in 95% ethanol at 1.28% concentration. 1ml of this solution diluted with 9 ml of BHI broth and the ethanol was removed by evaporating in a water bath (60°C) for 30 minutes,[4] and then serial two-fold dilutions were made in a concentration range of 2.5-640 µg/mL in a sterile test tube containing BHI broth. Two test tubes were considered as controls without BHA and microorganism, respectively. Inoculations of the tested yeasts were prepared from 24-hr BHI broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity and then diluted to 1×10^4 microorganisms per ml. each of the test tubes were inoculated with 0.1 ml of the spore suspension of each of the yeasts. All the tubes were incubated at 30-35°C for 24-48 hr. After incubation, each of the tubes shaking on a Vortex mixer and petri plates that were filled with SDA, were inoculated with 0.1 ml of them. All the SDA mediums were incubated at 37°C for 24-48 hr. To evaluate the effect of BHA on the tested molds, were used inoculations of them were prepared from 24-hr SDA cultures. The MIC was defined as the lowest concentration of inhibitor to prevent the growth of microorganisms.[9,10]

Antifungal effect of BHA in combination with different NaCl concentrations

To amplification the inhibitory effect of BHA, it was mixed with 2 and 4% NaCl concentrations. For this purpose, during the preparation of the BHI broth culture, was added the 2 and 4 gr of NaCl per 100 ml of broth.[10]

Antifungal effect of BHA at different pH conditions

To amplification the antifungal activity of BHA, during the preparation of the BHI broth, the pH of the medium was adjusted to 3.5 and 5.5 with 1M HC1 or 1M NaOH prior to autoclaving.[10]

Statistical analysis

Assays were performed in three replicates. Data were analyzed using a one-way of ANOVA tests procedure of SPSS software (version 17 for Windows, SPSS Inc., Chicago, IL, USA).

Results

The results of present research indicated that BHA was effective against all of the microorganisms tested in the range of 20 to 320 µg/mL (Table 1). BHA was more effective in the presence of NaCl compared to acidic conditions. The MIC values of BHA was significantly lesser in media containing 4% and 2% NaC1 ($p< 0.05$). *Rhizopus arrhizus RAK* 220 was the most susceptible fungi to NaCl concentrations. In the presence of 2% NaCl concentration, the MIC value of BHA for *Rhizopus arrhizus RAK* 220 was reduced to $\frac{1}{4}$. As well, our results showed that the decrease in pH values had synergistic effect on the inhibitory activity of BHA on some microorganisms ($p< 0.05$). Among the tests, *Geotrichum candidum* UUFH 110 was the most susceptible fungi to reduce the pH values, as at pH 5.5 and 3.5 the MIC values of BHA reduced to $\frac{1}{2}$ and $\frac{1}{6}$, respectively. In contrast, at different pH, the MIC values of BHA on *Candida albicans* CAL 512 not only not decreased but also increased.

Discussion

Effect of BHA on the tested molds and yeasts

This study confirmed that BHA is an effective inhibitor of the growth of several tested molds and yeasts (Table 1). The MIC values was from 20 µg/mL for *Candida albicans* CAL 512 to 320 µg/mL for *Penicillium spp* PSIR 12 and *Rhizopus arrhizus* RAK 220. Other researchers reported similar antifungal activity of BHA. Thompson examined the fungitoxic effect of BHA on germination of conidia from seven toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus,* and observed that the germination rate was delayed by 100 µg/mL and prevented by 200 µg/mL of BHA.[11]

Thompson also concluded that BHA was a more effective phenolic antioxidant than PP for inhibiting growth of several *Aspergillus, Penicillium* and *Fussarium* species on potato dextrose agar (PDA) and reported that MIC values ranging from 250-500 ppm. As well this researcher found that higher MIC values was for *Aspergillus* than for *Penicillium* and *Fusarium.*[12] Razavi-Rohani and Griffiths demonstrated that the MIC of BHA against *Fusarium graminearum* PM162, *Penicillium roqueforti* IMI 129267 and *Aspergillus flavus* MM3 was <12 µg/mL and for *Candida* *lipolitica* 1591 was 460 µg/mL.[7] Also Khan *et al.* reported that BHA as the most promising *in vivo* control agent of crown rot disease of bananas in combination with low concentrations of benzoic acid and PP.[13] Jay found that BHA as generally more inhibitory than BHT to bacteria and fungi. This research, suggested that, in general, higher concentrations of these inhibitors are required to inhibit mold growth in foods in contrast with culture media.[14] Arroyo examined the antifungal effects of four phenolic antioxidants (BHA, PP, BHT and PG) on several molds and yeasts and found that only BHA and PP has remarkable antimicrobial activity *in vitro*. In Arroyo study, completely inhibition for all fungal growth were seen at 500 ppm concentration. In that study *Aspergillus ochraceus* was able to grow at 200 ppm concentration with lag times of 10 days (> 8-9 days more than the controls). Arroyo report MIC values of BHA and PP on wheat flour agar ranged between 200 and 500 ppm. Also he found that BHA showed stronger antifungal activity than PP, especially at low concentrations (50-150 ppm).[4]

Table 1. MIC values of butylated hydroxyanizole (BHA) alone and in combination with different NaCl concentrations and at different pH conditions against tested fungi in SDA

Organisms	MIC (µg/ml)*				
	BHA	**BHA+Nacl 2%**	**BHA+Nacl 4%**	**BHA+pH 5.5**	**BHA+pH 3.5**
***Aspergillusniger* ASNG 1214**	80	60	15**	80	80
***Aspergillusflavus* ASNS 22**	160	80**	40**	160	80**
***Aspergillusfumigatus* ASN 2211**	160	80**	30**	160	160
***Penicilliumspp* PSIR 12**	320	160**	80**	160**	120**
***Rhizopusarrhizus* RAK 220**	320	80**	60**	160**	120**
***Candida albicans* CAL 512**	20	60	40	80	40
***Rhodotorularubra* RRN 1214**	80	80	40**	80	60
***Geotrichumcandidum* UUFH 110**	60	30**	10**	30	10**

* Values are means of three replicates

** indicates significant differences compared to BHA at $p < 0.05$.

Antifungal effect of BHA in combination with different NaCl concentrations

The results of present study indicated that the addition of NaCl (2 and 4%) improve the antifungal activity of BHA (Table 1). Our results showed that the resistance of all eight tested fungi (except *Candida albicans* CAL

512), were decreased against BHA, when 2 and 4% NaCl was present in the culture medium. The MIC values of BHA was significantly lesser in media containing 4% and 2% NaC1 ($p < 0.05$). *Rhizopus arrhizus RAK* 220 was the most susceptible fungi to NaCl concentrations. In the presence of 2% NaCl concentration, the MIC value of BHA for *Rhizopus arrhizus RAK* 220 was reduced to ¼. Other studies also emphasize the synergistic effect of NaCl on the antifungal activity of BHA. Mossel *et al.* reported that antimicrobial properties of BHA became more effective on microorganisms when the salt concentration was raised from 3 to 7%.[15] Davidson & Branen and Razavi-Rohani & Griffiths found that the antifungal effect of BHA was significantly increase in media containing 5% and 10% w/v NaCl, respectively. They also reported at 10% NaCl concentration, BHA completely inhibited the organisms tested.[7,16]

Antifungal effect of BHA at different pH conditions

The effect of pH on the antimicrobial activity of BHA is highly dependent upon the microorganisms tested. Bacteria are more sensitive to BHA as pH decreases.[16] Similarly, our results showed that the decrease in pH values from 7 to 5.5 and 3.5 had synergistic effect on the inhibitory activity of BHA on some microorganisms ($p < 0.05$). Antifungal effects of BHA on some tested fungi at pH 3.5 was greater than at pH 5.5. Among the tests, *Geotrichum candidum* UUFH 110 was the most susceptible fungi to reduce the pH values, as at pH 5.5 the MIC values of BHA reduced to ½ and at pH 3.5 reduced to one-sixth. In contrast, at different pH, the MIC values of BHA on *Candida albicans* CAL 512 not only not decreased but also increased. Pitt and Hocking demonstrated that most molds can grow in a wide range of pH (pH=3-8), but most of them prefer acidic pH.[17] Razavi-Rohani and Griffiths reported that the BHA was less effective on yeasts and molds when the pH increased to 7 or decreased to 3.5, except for *Candida lipolitica* 1591 which was inhibited by approximately the same concentration of BHA regardless of medium pH. As well they found that the highest concentration of BHA that could inhibit all of organisms at all the pH values, was 460 µg/mL.[7] Arroyo examined the *in vitro* antifungal efficacy of BHA and PP at the different pH levels and reported that a relatively low effect of pH on the antifungal activity of antioxidants was found.[4]

CONCLUSION

It is evident that BHA exert an important antifungal activity in culture media that can be greatly influence by environmental factors include dose, fungal species/strain, NaCl concentrations, pH values and type of substrate. BHA is more effective in the presence of NaCl concentrations compared to acidic conditions. Thus, the use of BHA in the foods that processed with salt compounds are very useful for controlling several fungal species. As well the effect of substrate on the antifungal efficacy of BHA is very significant.

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التأثيرات المثبطة للالأنيسول هيدروكسي على نمو العفن والخمائر في ظروف الأس الهيدروجيني مختلفة وتركيزات مختلفة من كلوريد الصوديوم

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الملخص

تم عرض النشاط المثبطة للالأنيسول هيدروكسي (BHA) وحده، وعند pH مختلفة (3.5 و5.5) وتركيزات مختلفة من كلوريد الصوديوم (2 و4٪) في 5 قوالب و3 الخمائر. تم استخدام اختبار أنبوب التقليدية لتحديد التركيز المثبط الأدنى (MIC). كان BHA فاعلاً ضد كل من الكائنات الدقيقة التي تم اختبارها في مجموعة من 320-20 ميكروغرام/ مل. تم تثبيط خفض درجة الحموضة وبتركيزات كلوريد الصوديوم. كان BHA أكثر فعالية في وجود كلوريد الصوديوم مقارنة مع الظروف الحمضية. وكانت قيم MIC من BHA أقل بكثير في وسائل الإعلام التي تحتوي على 4٪ و2٪ NAC1 (P <0.05). كان الرازبة الرزية RAK 220 الفطريات الأكثر عرضة لتركيزات كلوريد الصوديوم. في وجود كلوريد الصوديوم تركيز 2٪، وقيمة MIC من BHA لالرازبة الرزية RAK 220 تم تخفيض ل¼. وكان الانخفاض في قيم درجة الحموضة 7-5،5.5 و3.5 تأثير متناغم على النشاط المثبطة للBHA على بعض الكائنات الحية الدقيقة (P <0.05). بين الاختبارات، كان التيربية كانديدوم UUFH 110 الفطريات الأكثر عرضة للحد من قيم الرقم الهيدروجيني، ودرجة الحموضة 5.5 في خفض قيم MIC من BHA إلى ½ وعلى درجة الحموضة 3.5 خفضت إلى السدس. في المقابل، في درجة الحموضة المختلفة، القيم هيئة التصنيع العسكري من BHA على المبيضات البيض CAL 512 ليس فقط لم ينخفض بل زاد أيضا. وتبين هذه الدراسات أن الخصائص المضادة للفطريات من BHA يمكن أن تتأثر بشكل كبير بالعوامل البيئية.

الكلمات الدالة: المثبطة للالأنيسول هيدروكسي (BHA)، العفن، الخميرة، قيمة الرقم الهيدروجيني، وتركيز كلوريد الصوديوم.