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Evaluation of five chemical food preservatives for their antibacterial activity against bacterial isolates from bakery products and mango pickles

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ABSTRACT

Outbreaks of food-borne pathogens (mainly bacteria) continue to draw public attention to food safety. Several reports have demonstrated the efficacy of using chemicals to control the growth of food spoilage and food-borne pathogens. The objective of this study was to investigate antibacterial activity of five chemical food preservatives against food associated bacteria isolated from bakery product and pickles. Acetic acid was found to be very active against Bacillus subtilis, Bacillus megaterium, Bacillus sphaericus, Bacillus polymyxa and three Escherichia coli isolates with inhibition zones ranging between 20mm to 22mm followed by lactic acid (14mm to 18mm), benzoic acid (3mm to 18mm). Citric acid was partially inhibitory against B. subtilis (15mm) and three isolates of Staphylococcus aureus (15mm to 16mm). Sodium acetate was only active against B. megaterium (15mm), but was inactive against other selected food-associated Gram-positive and Gram-negative bacteria. These findings indicated that acetic acid could be used to inhibit the growth of bacterial food spoilage and food-borne pathogens and can be used to improve the safety of food products.

Key words: Agar well diffusion, antibacterial activity, chemical food preservative, food associated bacteria, zone of inhibition.

INTRODUCTION

Foods are not only of nutritional value to those who consume them but often are ideal culture media for microbial growth. Chemical reactions that cause offensive and sensory changes in foods are mediated by bacteria that use food as a carbon and energy source. Some of the major bacterial genera which cause food borne infection and intoxication include. Some bacterial toxins are mutagenic and carcinogenic and some display specific organ toxicity. The major target organs for these toxins in human are liver, kidney, nervous system and endocrine system [1,2]. The problem for the food industry is to fulfill the demands of minimum changes in food quality and maximum security [3]. Chemical additives have generally been used to combat specific microorganisms. Beth *et al.* [4] reported that organic acids, such as lactic, acetic and citric acid, also can enhance or contribute to the flavour of acidified or fermented food, such as sausage, cheese and pickles. A large number of chemicals have been described that show potential as food preservatives, only a relatively small number are allowed in food products, due in large part to the strict rules of safety adhered to by the Food and Drug Administration (FDA) and to a lesser extent to the fact that not all compounds that show antimicrobial activity *in vitro* do so when added to certain foods [1, 5]. To enhance the shelf life of foods, several chemical preservatives have been employed [6]. Currently, limited information is available on the activity of chemical food preservative on the growth inhibition of food-borne pathogens in food products. In lieu of the above justification, the present endeavor was to evaluate the antibacterial activity of five chemical food preservatives against food associated bacterial isolates.

EXPERIMENTAL SECTION

Bacterial isolates

Ten food-associated bacteria (7 Gram-positive and 3 Gram-negative) were isolated from bakery products and pickles. The five chemical food preservatives such as sodium acetate, citric acid, benzoic acid, acetic acid and lactic acid (selected on the basis of their applications in bakery products and pickles [1,7] were evaluated for their antimicrobial activity against these selected food-associated bacterial isolates (*Bacillus subtilis* I, *B. megaterium* I, *B. sphaericus, B. polymyxa, Staphylococcus aureus* I, *S. aureus* II, *S. aureus* III (Gram-positive), *Escherichia coli* I, *E. coli* II and *E. coli* III (Gram-negative) by agar well diffusion method [8,9].

Evaluation of chemical food preservatives for their antibacterial activity

Preparation of stock solutions

The stock solutions of chemical food preservatives, sodium acetate, citric acid, benzoic acid (1% w/v i.e., 0.1g chemical preservative dissolved in enough sterile distilled water to make the final volume 10ml), acetic acid and lactic acid (1% v/v i.e., 0.1ml chemical preservative dissolved in enough sterile distilled water to make the final volume 10ml) were prepared [10].

Antibacterial activity by agar well diffusion method

In agar well diffusion method, PCA plates were inoculated with 100µl of each food-associated bacterium adjusted to standardized inoculum (1.5×10^8 CFU/ml) in triplicates and spread with sterile swabs. Wells or cups of 8 mm size were made with sterile cork borer into agar plates containing the bacterial inoculum and the lower portion was sealed with a little molten agar medium. 100µl volume of the chemical preservative was poured into a well of inoculated plates. Sterilized distilled water was used as a control which was introduced into a well instead of chemical food preservatives. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar [11, 12]. After incubation for 24 hrs at 37^oC, the plates were observed. If antibacterial activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the chemical food preservative. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded if the zone of inhibition was greater than 8 mm [13]. The mean and standard deviation of the diameter of inhibition zones were calculated.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of acetic acid against food-associated bacteria

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation [14, 15, 16]. On the basis of good antimicrobial activity (measured by zone of inhibition against bacteria) shown by chemical food preservatives, acetic acid was chosen for determination of MIC and MBC. MIC and MBC of acetic acid were determined by macrodilution agar method [14, 15].

Macrodilution agar method

In the macrodilution agar method, a two-fold serial dilution of the acetic acid was prepared in sterile distilled water to achieve a decreasing concentrations (in percentage) ranging from 1.0 to 0.031% (v/v) in eight sterile tubes labeled 1 to 8. Sterile cork borer of 8.0mm diameter was used to bore well in the presolidified Mueller Hinton agar (MHA) plates and 100µl volume of each dilution was added aseptically into the wells made in MHA plates in triplicate that had food-associated bacteria seeded with the standardized inoculum (1.5 X 10^8 CFU/ml). 100µl distilled water introduced into the well in place of acetic acid was used as control. All the test plates were incubated at 37°C and were observed for the growth after 24 hrs. The lowest concentration of an extract showing a clear zone of inhibition was considered as the MIC.

RESULTS AND DICUSSION

The preservative properties of weak organic acids have been exploited by mankind for thousands of years. The antimicrobial activities of many different weak acid food preservatives have been well documented [17]. Organic acids such as acetic, lactic, and citric acids have been used to control microbial growth, improve sensory attributes and extend the shelf life of various food systems including poultry [18] and fish [19]. The use of any antimicrobial depends on several factors, such as desired effect, legal limits of use and effect on food. The effectiveness of organic acids as antimicrobials differ widely based on concentration, pH, molarity and the concentration of the nondissociated form [4].

Of the five chemical food preservatives (acetic acid, sodium acetate, benzoic acid, citric acid and lactic acid) tested for their antibacterial activity, acetic acid was found to be best antibacterial agent. The highest activity was found against *B. subtilis, B. megaterium, B. sphaericus, B. polymyxa* and all the three *E. coli* and *S. aureus* isolates (Table 1 and Figure 1). Rosenquist and Hansen [20] was studied the effect of acetic acid and lactic acid against *Bacillus* spp. such as *B. subtilis* and *B. licheniformis* isolated from bread also found acetic acid as the most effective inhibitor of bacteria.

Lactic acid was found to be the second best antibacterial inhibitor followed by benzoic and citric acid. Sodium acetate showed almost nil activity. Bell *et al.* [21] reported that by dipping the beef in 1.2% acetic acid for 10 seconds and refrigerating at 5^0C for 20 hr, *Salmonella typhimurium* could be reduced by 73.3%. They also showed that by dipping pork chop for 2 min in 1% acetic acid prior to be packed in vacuum container and stored at 2-4^{0}C for 6 weeks, *Enterobacteriaceae* found in mentioned trial was less than the one dipped in sterilized water. In addition, Bell *et al.* [22] compared between spray wash treatment utilizing 1% acetic acid and distilled water for 15 seconds on surface beef. They found that acetic acid could eliminate *E. coli, Listeria innocua* and *S. wentworth* with more efficacy than the treatment held by distilled water.

Leesmith [23] reported the effectiveness of acetic acid against *S. anatum* and found that acetic acid (1%) with pH was at 3.18 could inhibit the growth of *S. anatum*. Doores [24] reported that bacteria inhibited by acetic acid include *Bacillus* spp., *Clostridium* spp., *L. monocytogenes, P. aeruginosa*, *E. coli* and *S. aureus.*

Acetic acid commonly called vinegar is a mono carboxylic acid with a pungent odour and taste. It has antimicrobial capabilities due to its ability to lower the pH and cause instability of bacterial cell membranes [1]. Acetic acid has been shown to be effective against *E. coli* O157:H7, reducing this pathogen by 100 colony forming unit (cfu/g). It has also shown to reduce *Salmonella typhimurium* by 73 cfu/cm^2 on carcass tissue surfaces [25]. Concentration of 3% acetic acid was quite effective in reducing counts of *Enterobacteriaceae* in vacuum packaged beef stored for 6 weeks at 2.4°C. Acetic acid is generally regarded as safe for miscellaneous and general-purpose usage [5].

Sodium acetate was found to be the least antibacterial in action as it inhibited the growth of *B. megaterium* only out of the ten bacterial isolates tested. In addition to their suppressing effect on the growth of food spoilage bacteria, organic salts of sodium acetate, lactate, and citrate have been shown to possess antibacterial activities against various food-borne pathogens including *Staphylococcus aureus* and *Yersinia enterocolitica*, *Listeria monocytogenes*, *Escherichia coli* [26] as well as *Clostridium botulinum* [27]. Furthermore, these salts are widely available, economical, and generally ‘‘recognized-as-safe’’ [28]. Anders *et al.* [27] reported that sodium acetate is an effective inhibitor of rope forming bacteria (*Bacillus subtilis*) in baked goods which is in agreement with our findings.

Lactic acid has been found to be very active against all the *Bacillus* and partial activity against *E. coli*. Ibrahim *et al.* [29] who studied the antimicrobial activity of lactic acid (0.2%) on growth of *E. coli* 0157:H7 in the laboratory medium and carrot juice reported that lactic acid had potent activity against *E.coli* 0157:H7 in both laboratory medium and carrot juice. Our results are also in accordance with Alakomi *et al*. [30], who reported the antimicrobial activity of lactic acid against Gram-negative bacteria such as *E. coli* and *P. aeruginosa.* Our results also substantiate the observation of Doyle *et al*. [5] that lactic acid has potent inhibitory activity against *S. aureus* and spore-forming bacteria. Lactic acid, a weak acid, may occur in both a dissociated and an undissociated form depending on the pH. It is the undissociated form of lactic acid that inhibits bacterial growth. The inhibitory capacity of this acid lies in its reduction of pH to levels below which bacteria cannot initiate growth. Lactic acid is an excellent inhibitor of spore forming bacteria at pH 5.0 [5]. Anderson and Marshall [31] found that the combination of lactic and acetic acid in concentration of 100% was most effective in reducing spoilage bacteria.

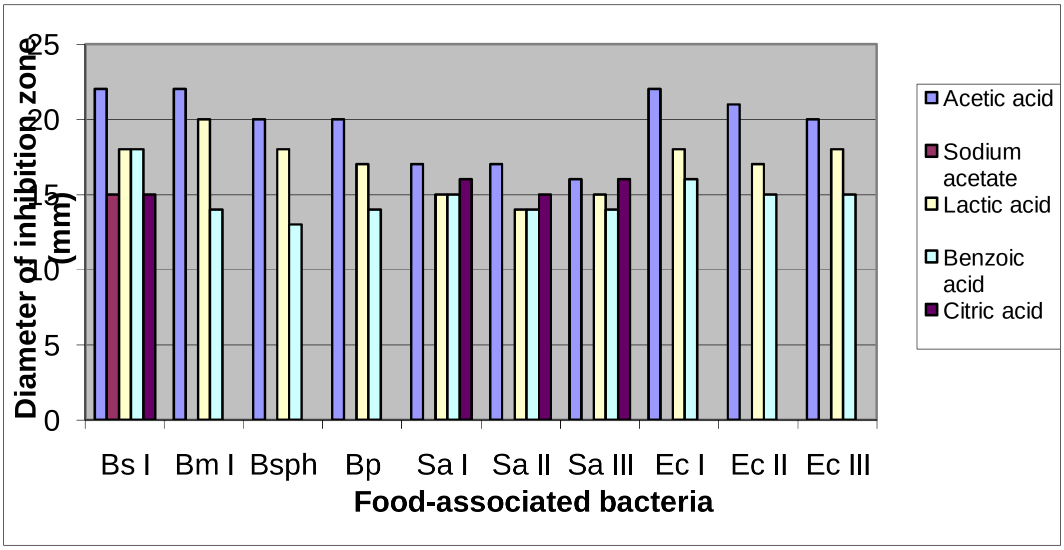
In the present investigation, citric acid has been found at the 4th position in its antibacterial activity showing inhibition of all the three isolates of *S. aureus* and *Bacillus subtilis* and showing no inhibition of Gram-negative bacteria i.e. *E. coli* isolates. Xiong *et al*. [32] had earlier reported the inhibitory activity of citric acid against *S. aureus* besides *Salmonella* sp. and *Clostridium botulinum*. In a study carried out by Sorrel [33], citric acid was investigated for its effect on inhibition of bacteria, yeast and molds and was shown to be inferior to lactic acid and acetic acid.

Table 1. Antibacterial activity of five chemical preservatives against food-associated bacteria by agar well diffusion method

Chemical food preservative	Diameter of inhibition zone (mm[a])									
	Bs I	**Bm I**	**Bsph**	**Bp**	**Sa I**	**Sa II**	**Sa III**	**Ec I**	**Ec II**	**Ec III**
Acetic acid	22±0.81[b]	22±0.81	20±0.57	20±0.57	17±0.37	17±0.37	16±0.37	22±0.81	21±0.81	20±0.81
Sodium acetate	15±0.81	NA	NA	NA	NA	NA	NA	NA	NA	NA
Lactic acid	18±0.37	20±0.81	18±0.57	17±0.37	15±0.57	14±0.37	15±0.37	18±0.31	17±0.37	18±0.37
Benzoic acid	18±0.57	14±0.37	13±0.57	14±0.37	15±0.57	14±0.37	14±0.57	16±0.81	15±0.81	15±0.81
Citric acid	15±0.37	NA	NA	NA	16±0.37	15±0.57	16±0.37	NA	NA	NA
Control (distilled water)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA- No activity; [a]-Values, including diameter of well (8mm), are means of the three replicate; [b] ±Standard deviation
Bs I- Bacillus subtilis I, Bm I- B. megaterium I, Bsph-B. sphaericus, Bp-B. polymyxa, , Sa I- Staphylococcus aureus I, Sa II- S. aureus II, Sa III- S. aureus III, Ec I-Escherichia coli I, Ec II-Escherichia coli II, Ec III-Escherichia coli II.

Fig. 1. Antibacterial activity of chemical food preservatives against food-associated bacteria by agar well diffusion method



Bs I- Bacillus subtilis I, Bm I- B. megaterium I, Bsph-B. sphaericus, Bp-B. polymyxa, , Sa I- Staphylococcus aureus I, Sa II- S. aureus II, Sa III- S. aureus III, Ec I-Escherichia coli I, Ec II-Escherichia coli II, Ec III-Escherichia coli III.

The antibacterial activity of citric acid is dependent on pH, concentration and anion effects [34]. Benzoic acid has been found to be partially active against all the Gram-positive and Gram-negative bacterial isolates during this study. Our results substantiate the findings of Doughari *et al.* [10], the spectrum of activity includes mainly enterobacteria and *Bacillus* spp. besides micrococci. Sofu *et al.* [35] had earlier reported the antibacterial activity of benzoic acid (0.1%)

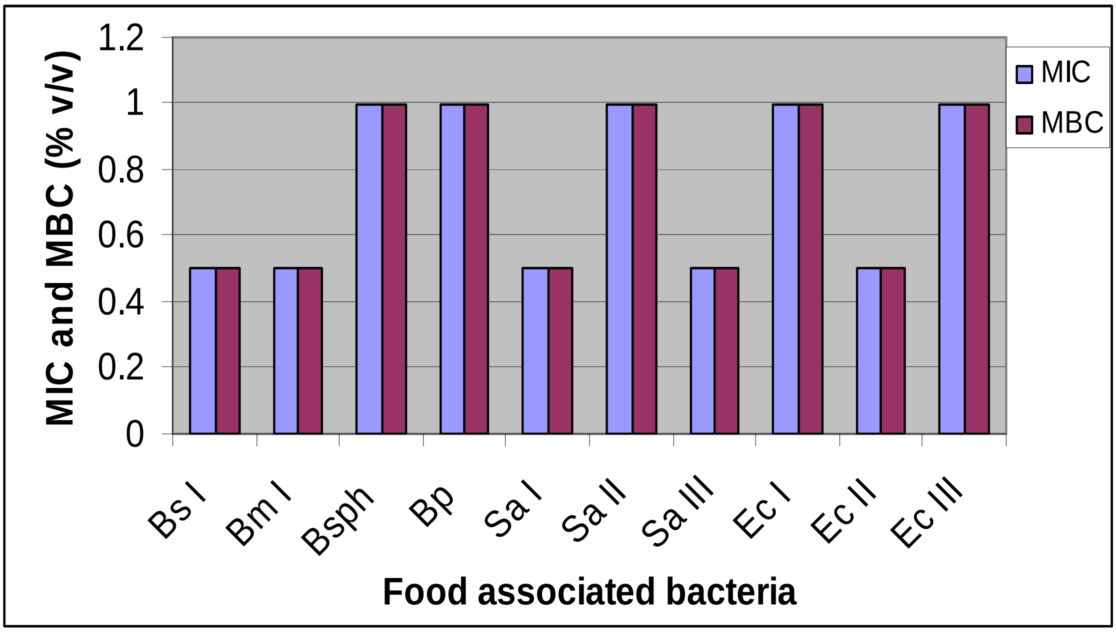
against *E. coli* in apple cider. According to Rajashekhara *et al.* [36], benzoic acid is also effective against another Gram-negative bacterium, *Listeria monocytogenes* at 1000µl/ml concentration. Dissociation of benzoic acid is strongly pH dependent and in its undissociated form it exhibits various antibacterial and antifungal activities [23].

Table 2. Minimum inhibitory concentration (MIC) and MBC of acetic acid against food-associated bacteria using macrodilution agar plate method

Food associated bacterial isolate	Concentration of acetic acid in percentage (v/v)							
	0.031	0.062	0.125	0.25	0.5	1.0	MIC	MBC
Bacillus subtilis	+	+	+	+	-	-	0.5	0.5
B. megaterium	+	+	+	+	-	-	0.5	0.5
B. sphaericus	+	+	+	+	+	-	1.0	1.0
B. polymyxa	+	+	+	+	+	-	1.0	1.0
Staphylococcus aureus I	+	+	+	+	-	-	0.5	0.5
S. aureus II	+	+	+	+	+	-	1.0	1.0
S. aureus III	+	+	+	+	-	-	0.5	0.5
Escherichia coli I	+	+	+	+	+	-	1.0	1.0
E. coli II	+	+	+	+	-	-	0.5	0.5
E. coli III	+	+	+	+	+	-	1.0	1.0

- No growth; + Growth

Fig. 2. Minimum inhibitory concentration (MIC) and MBC of acetic acid by using macrodilution agar plate method against food-associated bacteria



The *in vitro* minimum inhibitory concentration (MIC) of acetic acid which had been found to be the best antimicrobial agent of all the evaluated agents, was evaluated against the ten selected food-associated bacteria using macrodilution agar plate method (Table 2). The MIC values of acetic acid ranged between 0.5 and 1.0% (v/v) against food-associated bacteria. *Bacillus subtilis, B. megaterium, Staphylococcus aureus* isolates I, III, *E. coli* II were found to be the most sensitive which survived only upto 0.5% concentration of the acetic acid whereas *B. sphaericus, B. polymyxa, S. aureus* II, *Escherichia coli* I and III were found to be the most resistant bacterium which survived upto 1.0% concentration of the acetic acid. Thus, acetic acid was found to be the most effective with the lowest MIC of 0.5 against 5 bacterial isolates of the 10 tested isolates (Tables 2 and Fig. 2). The minimum bactericidal concentration (MBC), which was determined on the basis of the bacterial growth observed on the inoculated plates incubated at 37^0C for 24hrs, taken from the various concentrations of acetic acid (0.5 and 1.0% (v/v) as prepared for the MIC. The minimum bactericidal concentration (MBC) equaled the MIC of acetic acid which finally inhibited growth of food-associated bacteria. It can be concluded that acetic acid is more efficient antimicrobials than other organic acids tested and used to improve the safety of bakery products and pickles.

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