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Inhibition of Yeast in Commercial Pickle Brines

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Abstract

This study investigated the inhibition of yeasts in brines from fermented cucumber pickles using 2, 4-hexadienoic (sorbic), hexanoic and (E)-3-hexenoic acids. Native yeast population and chemical composition of commercial brines were analyzed and the minimum inhibitory concentrations of inhibitors on yeast growth were established. Commercial brines were treated with 100-350 ppm of 2, 4-hexadienoic (sorbic), hexanoic and (E)-3-hexenoic acids individually and at 2.5 to 10% salt (sodium chloride) concentrations. Yeast populations in the treated brines were monitored for 30 days of incubation. Hexanoic and (E)-3-hexenoic acids at 350 ppm caused reduction in yeast populations by about 4 and 2 log CFU/ml, respectively, within 24 hours of treatment. However, when brines were treated with 2, 4-hexadienoic acid at salt concentrations of 7.5 to 10%, there were no significant differences noted in yeast inhibition between the three acids. Hexanoic and (E)-3-hexenoic acids at 200 ppm caused longer lasting inhibitory effects (30 days) on yeasts than the traditionally used 2, 4-hexadienoic acid (10 days) in fermentation brine. Thus, the hexanoic and (E)-3-hexenoic acids are potential alternatives to 2, 4-hexadienoic acid for controlling yeasts during storage of spent cucumber fermentation brines.

Keywords: yeast inhibition, cucumber pickle brine, 2, 4-hexadienoic acid, hexanoic acid, hexenoic acid

1. Introduction

Commercial fermentation of cucumbers is mainly done in open tanks filled with freshly prepared or recycled brine solution containing 6-8% sodium chloride. The salt in brine solutions helps to preserve firmness of the fruits, prevent growth of spoilage bacteria and select homo-fermentative lactic acid bacteria. (Fleming, 1982). However, certain yeasts such as *Hansenula, Torulopsis, Saccharomyces, Candida, Debaromyces,* and *Rhodotorula* can grow in the spent brines and drastically affect the quality of cucumber pickles (Etchells, Costilow, Bell, & Rutherfords, 1961). Other yeasts such as the film forming *Pichia anomala* are also reported in pickled vegetables (Hung & Kyung, 2006; Pitt & Hocking, 2009). Although commonly used salt concentrations can selectively promote lactic acid bacterial growth (Etchells & Jones 1943), they may not affect the survival of salt tolerant yeasts. Failure to inhibit growth of yeasts, molds, certain bacteria and their enzymes in brines can affect the color, flavor and texture of the pickled product wherein the growth of yeasts in cucumber fermentation tanks can specifically reduce lactic acid, increase brine pH, and increase carbon dioxide production leading to bloater formation (Jones, Etchells, Veerhoff, & Veldhuis, 1941). Although the problem of yeast growth in commercial fermentation tanks is typically addressed by adding 2, 4-hexadienoic (sorbic) acid (Costilow, Ferguson, & Ray, 1955; Costilow, Coughlin, Robbins, & Hsu, 1957), the preservative effect of this compound is usually temporary because of its susceptibility to microbial degradation causing more than 50% loss of the added amounts within a month (Gordon & Lewis 1958). This degradation of 2, 4-hexadienoic acid is also known to be influenced by exposure to sunlight since fermentation tanks are left open to reduce the growth of surface yeasts during fermentation and bulk storage (Saxby, Stephens, & Reid, 1982). Thus, alternatives to sorbic acid need to be evaluated for their efficacy to inhibit growth of yeast in cucumber fermentation brines.

Majority of natural flavoring agents are also known to contain broad range of compounds imparting antimicrobial properties against different food pathogenic and spoilage causing microorganisms including *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica* and yeasts such as *S. cerevisiae, Candida albicans, Debaryomyces hansenii* (Friedman, Henika, & Mandrell, 2002; Suppakul, Sonneveld, Bigger, & Miltz 2008; Gutiérrez, Escudero, Batlle, & Nerín, 2009; Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011). The yeast inhibiting properties of many flavoring agents or essential oils can be attributed to their natural components such as eugenol present in oils of many spice, cinnamon leaf, clove bud and leaf, and

cinnamamic aldehydes present in cinnamon bark oil, cassia oil (Kuorwel et al., 2011). These compounds are also known for their antimicrobial properties in acid foods (Jay & Rivers, 1984) indicating their use as preservatives in cucumber fermentation brines. However, the essential oil components to be used for preservation of cucumber pickle should be effective under high salt concentrations of the fermentation brines. Due to the vast number of existing natural or chemical preservatives, the selection of a natural compound inhibiting yeast in high salt containing cucumber fermentation brines can be done by confirming their lasting effectiveness and structural similarity to existing stable compounds. Hexanoic and (E)-3-hexenoic acid are natural flavoring agents and are expected to be more stable and effective as they have no or one double bond respectively when compared to the two double bond containing 2, 4-hexadienoic acid. Furthermore, these compounds are considered as weak acid preservatives that are effective at acidic pH (Eklund, 1980) and lesser double bonds of these compounds could make them more stable than sorbic acid. Thus, the objectives of this study were to evaluate inhibition of yeast growth in commercial spent cucumber fermentation brines using hexanoic, (E)-3-hexenoic and 2, 4-hexadienoic acids and also to compare their effectiveness at different salt concentrations.

2. Method

2.1 Preparation of Spent Brines and Test Compounds

Spent brines were obtained from commercial pickle manufacturers. Test compounds for inhibition of yeast growth in commercial spent brine were obtained from Sigma-Aldrich Chemical Company supplied at 99.9% purity by the manufacturer. Hexanoic and (E)-3-hexenoic acids that were insoluble in water were emulsified before use by sonicating with Tween-20 at 1/0.5 ratio and by gradually adding deionized water while sonicating. The final volume of emulsion was adjusted to 100 ml by slowly adding deionized water. Total sonicating time varied from 1 to 2 hours depending on each chemical and each emulsion was used immediately for subsequent study. Different concentrations (0, 25, 50, 100, 200, 300, and 400 ppm) of the oil emulsion were prepared in 100 ml of commercial recycled brine using a 20,000 ppm stock solution for each chemical, thus even the highest concentration of 400 ppm used in treatments did not have a major dilution on the brine samples. The samples were placed in disposable plastic cups and lightly covered by plastic Petri dishes to prevent evaporation. The test samples were incubated in dark at 25 ± 2°C for 30 days.

2.2 Chemical Analysis of Recycled Brines

Spent brines were assayed for NaCl, titratable acidity, pH and calcium as previously described (Buescher & Burgin, 1988). Sorbic acid content in the spent brines was measured by a modified protocol of Ziemelis and Somers, (1978) as previously described by Buescher and Hamilton (2000).

2.3 Yeast Enumeration from Brine Samples

Yeast populations of the brine samples were estimated by dilution plating wherein the recycled brine was serially diluted in sterile 0.1% buffered peptone water (Difco Co., Detroit, MI) and 1ml of each dilution was plated on yeast and mold (YM) Petrifilms (3M Co., St. Paul, MN). The plates were incubated for 5 days at 25 ± 2°C. Plating and counting techniques were done as described in YM petrfilm brochure. Surface yeast was determined by the appearance of white film on surface of brine. Confirmation of yeast cells was made by observation of the film layer suspension prepared in 0.1% buffered peptone water under microscope using 40X magnification. Enumeration of initial yeast population in recycled brine was done before each test and necessary adjustment of yeast population was needed to be done for some brine obtained from processing companies as the population of yeast varied from tank to tank.

2.4 Inhibition of Yeast Growth by Hexanoic, (E)-3-hexenoic, and 2, 4-hexadienoic Acids

Yeast inhibition by use of hexanoic, (E)-3-hexenoic, and 2, 4-hexadienoic (sorbic) acids was compared at 350 ppm concentration of each compound prepared in 100 ml of recycled brine. Untreated recycled brine samples were used as control. For each treatment, viable yeast population was measured at 0, 24, and 48h. Time of film yeast appearance on the surface of brine was recorded during the 30-day observation period. Another set of treatments was established the same way as described above except that the initial yeast population in the brine was 10^5 CFU/ml and concentrations from 0 to 350 ppm of each chemical were tested for yeast inhibition.

2.5 Influence of Sodium Chloride on Yeast Inhibition by Hexanoic, (E)-3-hexenoic, and Sorbic Acids

A commercial recycled brine sample was diluted using deionized water to contain 2.5% NaCl, and then adjusted to NaCl concentrations to 5, 7.5, and 10%. This was done to start with same base brine solution for the experiment on testing the effectiveness of Hexanoic, (E)-3-hexenoic or 2, 4-hexadienoic acid (each at 350 ppm) under different salt concentrations. In addition, pH was adjusted to 3.3 in all treatments using HCl. The time for film yeast first appearance on the brine surface and viable yeast population were recorded.

2.6 Statistical Analysis

The values of Log CFU/ml of yeast populations and the days of appearance averaged from three independent replications were subjected to one way analysis of variance (ANOVA) by Statistical Analysis Software (SAS 9.2, SAS Institute Inc. Cary, NC), and mean comparisons were done using Tukeys significance grouping where p values of <0.05 were considered statistically significant. Data plotting was done using SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA)

3. Results and Discussion

The commercial spent brines used for yeast inhibition experiments contained 8.8% to 9.4% NaCl with pH of 3.30-3.50, and 0.69% to 0.77% lactic acid. The native yeast population of the spent brine was 10^4 to 10^5 CFU/ml and when the yeast population was found to be lower than this level, the brine was incubated at 25°C until it reached at least 10^4 CFU/ml. Although the identification of yeast species was beyond the scope of this study, the growth pattern and colony types were found to be similar when plated on YM plates and as determined using a microscope at 40X magnification.

The effects of hexanoic acid, (E)-3-hexenoic acid, and 2, 4-hexadienoic acid on inhibition of film yeast growth in cucumber recycled brine was tested. Even though these acids were described as unpleasant flavors (Fenaroli, 2002), they did not cause an objectionable odor in recycled brine at concentrations as high as 350 ppm. The minimum inhibitory concentrations (MIC) for yeast inhibition by hexanoic acid and hexenoic were 200 and 350 ppm, respectively, and they prevented film yeast growth for more than 30 days at 200 ppm, whereas same amount of sorbic acid prevented film yeast for only 10 days (Table 1). In the control brine where no inhibitor was added, film yeast growth occurred within 2 days.

Table 1. Effect of hexanoic, hexenoic, and 2, 4-hexadienoic (sorbic) acid concentrations on inhibition of yeast in spent cucumber fermentation brine

Treatment	Time (days) for appearance of yeast						
	Treatment Concentration (ppm)						
	0	25	50	100	200	300	350
Control	2	-	-	-	-	-	-
Hexanoic acid	-	-	-	-	>30	>30	>30
Hexenoic acid	-	2	3	4	>30	>30	>30
2,4-hexadienoic acid	-	2	2	3	10	>30	>30

Before identifying the optimum concentrations of hexanoic acid, (E)-3-hexenoic acid and 2, 4-hexadienoic acids on the yeast population in commercial recycled brine, the chemicals were first tested at 350 ppm concentration against a known yeast population. A brine solution containing 4.5 log CFU/ml of yeast population with similar pH, NaCl, and lactic acid concentration was used for this study along with a control brine without any of the added inhibitory compounds. The results indicated that, within 24 hours of application, hexanoic acid was the most effective among the three test chemicals reducing the yeast population by about 3.5 Logs CFU/ml, whereas (E)-3-hexenoic acid and sorbic acid showed reduction by 1 and less than 1 log CFU/ml respectively (Figure 1). After 48 hours of treatment, yeast population in the brine samples treated with the three acids were significantly less ($p<0.05$) in comparison to the control samples. Further, identification of optimum inhibitory concentrations of hexanoic, (E)-3-hexenoic, and sorbic acid on growth of film yeast and yeast population was done using 100-350 ppm concentrations of the compounds in the recycled brines that were adjusted to contain initial yeast population of 7.3×10^5 CFU/ml. As shown in Figures 2 (A), 2 (B) and 2 (C), the yeast inhibition by adding 100 ppm of hexanoic, and (E)-3-hexenoic acids did not differ significantly with the control ($p<0.05$). At about 200 ppm, hexanoic acid and sorbic acid reduced the yeast population after 2 days of treatment to 10 CFU/ml and continued to suppress the number of yeasts for the following days. On the other hand, 200 ppm (E)-3-hexenoic acid was not effective in reducing the number of yeast, but it effectively prevented yeast from developing. At 350 ppm, hexanoic acid reduced the yeast population by 3 log CFU/ml within 2 days, while (E)-3-hexenoic acid and sorbic acid reduced 2 log CFU/ml. Sorbic acid continued to reduce the yeast population by 2 more log CFU/ml in the following 2 days, while hexanoic acid and (E)-3-hexenoic acid did not further reduce the number of yeasts.

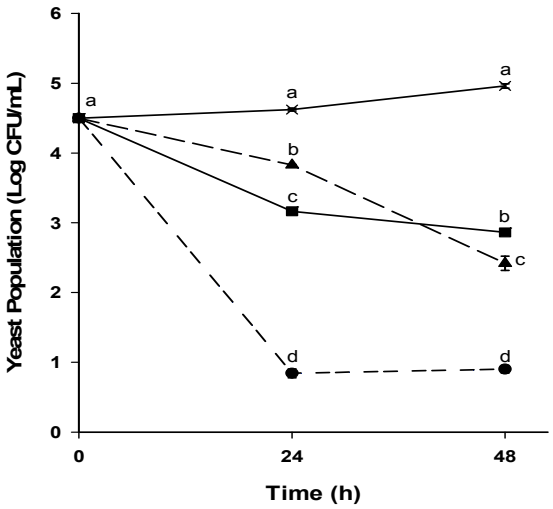
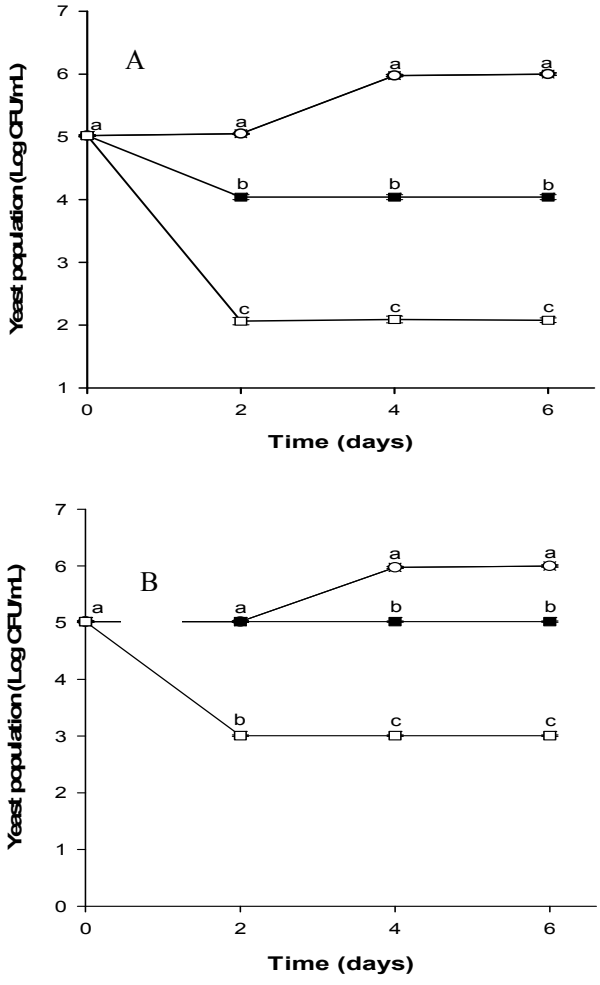


Figure 1. Inhibition of indigenous yeast in commercial spent brine by 350 ppm hexanoic acid, (E)-3-hexenoic acid, or 2, 4-hexadienoic acid. The brine contained 8.83% NaCl, 3.34 pH, 0.77% lactic acid and initial yeast population of 4.8 x 10^4 CFU/ml. The mean values at each time of measurement labeled with different letters indicate significant difference ($p < 0.05$). Symbols indicate Control (-x-), Hexanoic acid (-●-), Hexenoic acid (-■-) and 2, 4-hexadienoic acid (-▲-)



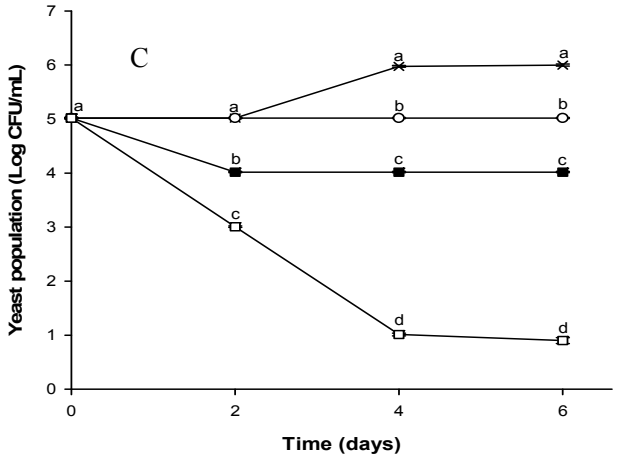


Figure 2. Inhibition of yeast population in commercial brines added with hexanoic acid (A), (E)-3-hexenoic acid (B), and 2,4-hexadienoic acid (C) at 100-350 ppm concentrations. Initial yeast count in the brine was at $7.3x10^5$ CFU/ml, with 8.9% NaCl and 3.3 pH. The mean values at each day of measurement with different letters indicate significantly difference ($p<0.05$). Symbols indicate Control (-x-), 100ppm (-○-), 200ppm (-■-) and 300ppm (-□-)

The effects of sodium chloride concentration on yeast inhibition by hexanoic, (E)-3-hexenoic and sorbic acids in commercial recycled brine was also tested. Although sodium chloride is added as preservative in spent brine, addition of NaCl alone at concentrations of less than 7% may not be effective for yeast inhibition (Yang & Buescher, 2000). Furthermore, even at concentration as high as 20%, NaCl alone may not be very effective in inhibition on most of the fungi (Kurita & Koike, 1982) in brine solutions. Thus, the effect of NaCl concentration on yeast population of brine treated with 350 ppm of hexanoic acid, (E)-3-hexenoic acid, or sorbic acid was examined to test their efficacy in commercial brine (Figure 3). Hexanoic acid and sorbic acid both inhibited yeast growth for more than 30 days in brine containing 5, 7.5, and 10% NaCl and differed significantly with control ($p<0.05$). However, NaCl at a concentration of 2.5% failed to exhibit any observable anti-yeast effect with any of the chemical treatments or control. Results indicated that 350 ppm of inhibitors effectively inhibited yeast growth in samples containing 5% or more NaCl. The anti-yeast effect of these chemicals in brine with 2.5% NaCl was substantially less than that in 5% or more NaCl. Apparently, NaCl at concentration of 5% or more substantially increased effect of hexanoic, (E)-3-hexenoic, and sorbic acid in yeast growth inhibition.

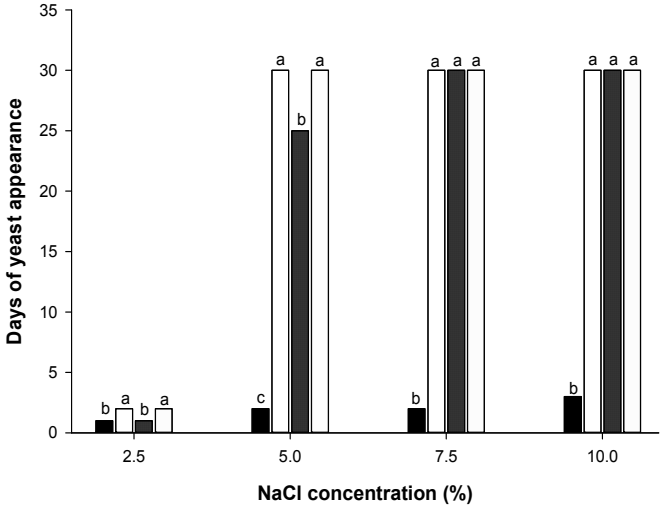


Figure 3. Effect of NaCl concentrations in spent cucumber fermentation brine on yeast inhibition by control (no inhibitor) (-▉-), 350 ppm hexanoic acid (-□-), 350 ppm hexenoic acid (-▒-) or 350 ppm 2, 4-hexadienoic acid (-▒-) at pH 3.3 and 25±2°C incubation temperature. The mean values of days for appearance of yeast at each NaCl concentration with different letters indicate significant difference ($p<0.05$)

4. Conclusions

Yeasts were abundant in commercial spent cucumber fermentation brines and increased in number unless treated with hexanoic, hexenoic or 2, 4-hexadienoic (sorbic) acids. Brines containing 200 ppm of either of the acids prevented yeast growth and at 350 ppm they substantially reduced the yeast population. Inhibition by the acids was minor in brine containing 2.5% NaCl, but very effective when the NaCl concentration was 5% or higher. It appears that hexanoic or hexenoic could be used instead of sorbic acid to prevent yeast growth during storage of spent cucumber fermentation brine.

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