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RESEARCH NOTE

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Inhibition of Yeast Film Formation in Fermented Vegetables by Materials Derived from Garlic Using Cucumber Pickle Fermentation as a Model System

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Abstract Film-forming yeasts generate an undesirable yeasty flavor in fermented vegetables such as *kimchi* in the presence of oxygen. Antimicrobial materials including garlic oil (GO), heated garlic (HG), and allyl alcohol (AA) were investigated for use as alternative natural food preservatives to inhibit the growth of film-forming yeasts in fermented vegetables. Using the fermentation of cucumber pickles as a model system, GO, HG, and AA were effective in preventing film formation at concentrations of 0.006, 3.0, and 0.02%, respectively. The effectiveness of HG in preventing the growth of a film yeast, *Hansenula anomala*, was not influenced by pH, while that of potassium sorbate, a typical anti-yeast food preservative, was highly dependent on pH. All tested materials were effective when added at the beginning of fermentation due to their negligible inhibitory activity toward lactic acid bacteria.

Key words: film yeast, *Hansenula anomala*, garlic oil, heated garlic, allyl alcohol

Introduction

Yeasts active during the fermentation process of vegetables are divided into two groups; the subsurface (fermentative) type and the surface (oxidative) type. The latter forms a yeast film aerobically in fermented pickled vegetables. When a considerable amount of sugar is left unfermented after lactic acid fermentation, fermentative subsurface type yeasts utilize the sugar to generate gases and cause a yeasty flavor (5). Oxidative surface type yeasts do not ferment sugars anaerobically and oxidize primary fermentation products such as lactic acid only in the presence of oxygen, resulting in a pH rise, and a yeasty off-flavor. Off-flavor caused by surface film yeasts is a major concern regarding *kimchi* quality. In addition, higher pH may support the growth of putrefactive bacteria, causing bad odor and potential spoilage. Subsurface type yeasts do not seem to be as problematic as oxidative film-forming yeasts (5). Film yeast growth during vegetable fermentation results in the oxidation of lactic acid and leads to spoilage if insufficient acids remain to inhibit the growth of undesirable bacteria. Although yeast growth is easily controlled by chemically synthesized preservatives such as sorbic acid and its salts, consumers prefer products without artificial chemical preservatives (14, 15).

Edible plant materials are becoming popular as natural food preservatives in food processing. Conner and Beuchat (4) investigated the effects of essential oils from plants on the growth of food spoilage yeasts and concluded that such essential oils impair a variety of enzyme systems, including those involved in the production of cellular energy and the synthesis of structural components. O'Gara *et al.* (12) reported that garlic oil (GO) consists of sulfides such as diallyl trisulfide, diallyl tetrasulfide, diallyl pentasulfide, and diallyl hexasulfide which possessed antimicrobial activity against *Helicobacter pylori*. Interestingly, essential oils of garlic and onion and their constituent sulfides are potent inhibitors of yeasts, but are very weak inhibitors of bacteria (7). These characteristics make GO and its constituent good candidates for the suppression of film yeasts in vegetable fermentations. GO is obtained by steam distillation of crushed garlic (13). Among plants of the *Allium* species, garlic and onion have been the most extensively studied for their antimicrobial activities. Many recent investigations have shown that extracts from *Allium* bulbs inhibit the growth and respiration of pathogenic fungi and bacteria. Saleem and Al-Delaimy (16) showed that the aqueous extract of fresh garlic bulbs at levels of 3, 5, and 10% inhibited the growth of *Bacillus cereus* on nutrient agar plates by 31.3, 58.2, and 100%, respectively. Growth of various fungi was also inhibited by an aqueous extract of garlic at levels as low as 1:1024 (11). Essential oils of garlic and onion containing various sulfides might be potential natural preservatives, similar to fresh garlic and mustard which are already used as preservatives in some foods (1, 2, 8). Using the active compounds of garlic and onion for controlling yeast growth in food is limited, however, by the flavor and aroma characteristics they often impart at low concentrations. Recently, Choi and Kyung (3) reported that allyl alcohol (AA), generated through thermal decomposition of alliin in garlic, was a potent anti-yeast compound. AA is the principal anti-yeast compound of heated garlic (HG), which has also been reported to inhibit yeast and bacterial growth (6, 8).

The objective of this study was to investigate the possibility of using GO, HG, and AA to prevent yeast film formation in fermented vegetable products using cucumber pickle fermentation as a model system.

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Materials and Methods

Materials Cucumber, garlic, and salt used for cucumber pickle fermentation were purchased from a local market in Seoul, Korea. Garlic oil (GO) from Grupo Tecnaal. Co. (Zapopan, Mexico) was donated by Hyangwon Spice Co. (Sungnam, Gyeonggi, Korea). Tween 80 and allyl alcohol (AA) were purchased from Aldrich Chem. Co. (Milkwaukee, WI, USA), and Sigma Chemical Co. (St. Louis, MO, USA), respectively. Potassium sorbate (PS) was purchased from Duksan Pure Chemicals Co., Ltd. (Gyeonggi, Korea).

Microorganisms Microorganisms used in this study included *Lactobacillus plantarum* LA 97, *L. plantarum* ATCC 14917, *Leuconostoc mensenteroides* subsp. *mensenteroides* ATCC 8293, *L. mensenteroides* subsp. *dextranicum* ATCC 19255, *Hansenula anomala, Pichia membranefaciens* Y20, *Saccharomyces cerevisiae* ATCC 4126, and *Candida utilis* ATCC 42416. These strains were a gift from Professor Henry P. Fleming (Food Fermentation Laboratories, USDA/ARS, North Carolina State University, Raleigh, NC, USA). *Pediococcus pentosaseus* and *Streptococcus feacalis* were obtained from a culture collection in the Microbiology Laboratory of the Department of Food Science, Sejong University. *Lactobacillus brevis* KFCC 35464 IFO 3345 was obtained from the Korean Federation of Culture Collection (KFCC, Seoul, Korea). *Zygosaccharomyces rouxii* KCCM 50523 was purchased from the Korea Culture Collection of Microorganisms (KCCM; Seoul, Korea); *Candida albicans* KCTC 7965 was purchased from the Korea Collection for Type Culture (KCTC; Daejeon, Korea).

Stock cultures of bacteria and yeasts were stored at -64°C in basal media containing 16% glycerol. The basal media were MRS broth (Difco Laboratories, Detroit, MI, USA) for lactic acid bacteria (LAB), and YMPG broth (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, and glucose 1%, Difco Lab.) for yeasts. Frozen cultures were streaked onto an agar plate containing the same medium used for growth, and an isolated colony was picked and subcultured at least twice in growth medium before using a 24 hr cultures for the final inoculation of bacteria and nonxerotolerant yeasts, and a 48-hr culture for xerotolerant yeasts. Yeasts were grown aerobically by shaking at 150 rotations per min (KSI-200L shaker; Korea Environmental Control Co., Ltd., Gyeonggi, Korea). Five μL of a 10-fold diluted aliquot of bacterial seed (or yeast seed) culture were inoculated into 5 mL of appropriate broth in glass culture tubes (16×150 mm) and statically incubated. The number of viable cells was determined by spiral plating (Spiral Autoplate System, Spiral Biotech Inc., Bethesda, MD, USA) on agar plates (Difco Lab.), incubating for 24 to 48 hr and counting the resulting colonies. Viable cell counts were then expressed as colony-forming units (CFU)/mL. All growth studies were performed at 30°C.

Cucumber pickle fermentation as a model system Cucumbers were purchased, trimmed, and washed with tap water. Washed cucumbers were cut into 5-7 cm segments which fit into fermentation containers with two garlic cloves. These were then used as a supplemental source of natural microorganisms including LAB (17). Since film formation by natural film yeasts appeared irregularly during preliminary cucumber pickle fermentation experiments, *H. anomala*, a film yeast, was added to the containers at a ratio of about 100 cells/L. Cucumbers were fermented at 30°C for 14 days in four different of concentrations (final) of sodium chloride: 1.2, 2.4, 3.6, and 4.8%.

Heated garlic (HG) extract preparation Peeled garlic cloves were blanched in boiling water for 10 min to inactivate alliinase. The boiled garlic was cooled with flowing tap water, blended (Waring Blender, New Hartford, CT, USA) with an equal weight of sterilized distilled water, and centrifuged at 15,000×g (HMR-2001V; Hanil Industrial Co., Incheon, Korea) for 20 min to remove insoluble residue. The supernatant was dispensed into screw-capped glass tubes and autoclaved at 120°C for 45 min. The HG extract was centrifuged at 20,000×g for 20 min and sterile filtered. HG extract was added into sterile glass tubes containing appropriate culture broths to make test media with the desired concentrations.

Cucumber juice (CJ) preparation Cucumber was washed and blanched by steam at ambient pressure for 10 min. The steamed cucumbers were cooled with flowing tap water, blended, and centrifuged at 7,500×g for 20 min to remove insoluble residue (9). The supernatant was dispensed into screw-capped plastic bottles and stored in the freezer (-64°C) until use. The frozen cucumber juice was thawed and centrifuged at 20,000×g for 20 min, and then the supernatant was mixed with a 6% NaCl solution to make a 3.6% NaCl test solution and filter-sterilized before use. CJ and YMPG broths were adjusted to the desired pH (3, 4, 5, 6, and 7) with lactic acid (10%) or 1 N NaOH to compare the relative effectiveness of HG and PS in inhibiting *H. anomala* at different pHs.

Allyl alcohol (AA), garlic oil (GO), and potassium sorbate (PS) stock solution preparation AA and PS were dissolved in the appropriate growth medium to make 1.0 and 0.1% stock solutions, and filter-sterilized. GO was dissolved in the appropriate growth medium with 0.5% Tween 80 as a surfactant to make a 0.1% stock solution which was filter-sterilized.

Minimum inhibitory concentration (MIC) determination HG and stock solutions of AA, GO, and PS were diluted with sterile culture broth to give the desired concentrations. Medium with the desired test concentrations of inhibitory materials and 3.6% NaCl was inoculated with microorganisms to give an initial cell concentration between 10^4-10^5 cells/mL and incubated at 30°C for 24 hr for bacteria, and 48 hr for yeasts. The sensitivity of the test microorganisms was expressed as the MIC in %. Experiments were performed in duplicate and the lowest values were recorded as MICs. A complete absence of surface film based on optical observation after the incubation period was regarded as non-film formation.

Results and Discussion

Inhibition of film formation in fermenting cucumber pickles by garlic materials All three materials tested were effective in preventing yeast film formation using cucumber pickle fermentation as a model system. Garlic oil (GO), heated garlic (HG), and allyl alcohol (AA) prevented yeast film formation at concentrations of 0.006, 3, and 0.02%, respectively, for 30 days of pickle fermentation (Table 1). Potassium sorbate (PS) was effective at a concentration of 0.02%. Film first appeared on the 3rd day of fermentation in untreated control samples. GO is known to be strongly antimicrobial because of the presence of various kinds of sulfides (1, 2). The antimicrobial activity of GO was stable during storage for up to one month at relatively high temperature (37°C) and was not affected by pH (7). These properties make GO a promising natural preservative in foods that are spoiled by yeasts. HG also inhibited yeast film formation and showed stable anti-yeast activity (8) which also was unaffected by pH (Fig. 1). Furthermore, HG has a much weaker odor (threshold level not studied) than GO which has a very strong garlic odor.

Inhibitory effects of GO, HG, and AA against lactic acid bacteria and yeasts in different media The potency of growth inhibition of GO, HG, and AA was tested against various lactic acid bacteria (LAB) and yeasts in cucumber juice (CJ), MRS broth for LAB, and YMPG broth for yeasts. The results showed that the MIC of GO against LAB including *L. plantarum* LA 97, *L. plantarum* ATCC 14917, *L. brevis* KFCC 35464, *S. feacalis, P. pentosaceus, L. mensenteroides* subsp. *mensenteroides* ATCC 8293, *and L. mensenteroides* subsp. *dextranicum* ATCC 19255 in both CJ and MRS broth, was very high, being over 0.1% (Table 2). These results confirmed that GO did not have a strong inhibitory effect against LAB, including *L. mensenteroides* LA10, *P. pentosaceus* LA3, and *L. plantarum* LA 97 as previously reported (7). In both media, the LAB were very resistant to HG as shown by a MIC of over 50%, which was several orders of magnitude higher than the MIC of HG against yeasts (Table 2).

AA also showed strong inhibitory activity against various yeasts (*H. anomala, P. membranefaciens* Y20, *C. utilis* ATCC 42416, *C. albicans* KCTC 7965, *S. cerevisiae* ATCC 4126, *and Z. rouxii* KCCM 50523). The MIC of AA against various yeasts in CJ and YMPG broth ranged from 0.0002 to 0.015%, respectively, which was several hundreds times lower than the MIC seen against LAB (Table 2). These results indicate that garlic materials are very strongly inhibitory against yeasts including film yeasts such as *H. anomala, P. membranefaciens, and Z. rouxii*. On the other hand, garlic materials were only very weakly inhibitory against LAB. Therefore, all three materials derived from garlic can be added at the beginning of fermentation to inhibit yeasts without harming the desirable lactic acid fermentation of vegetables.

CJ was regarded as a better growth medium for various yeasts than YMPG broth judging from the greater MICs in CJ medium relative to those in YMPG broth (Table 2). This suggests that CJ naturally contains some unknown components that stimulate the growth of yeasts. Amino acids, peptides, bases, and nucleosides naturally present in plants have been shown to stimulate microbial growth (18, 19). *H. anomala,* a film yeast, is more resistant to AA than other tested yeasts, with the MIC being 0.006 and 0.015% in YMPG broth and CJ, respectively (Table 2). *P. membranefaciens*, another type of film yeast, was the most sensitive to the inhibitory activity of garlic materials with MICs for GO, HG, and AA in YMPG broth of 0.0004, 0.2, and 0.0002%, respectively.

Because GO, HG, and AA showed potent growth inhibition toward yeasts while showing weak inhibition against LAB, these garlic materials can be effectively utilized as natural antifungal food preservatives in fermented vegetables throughout the fermentation and storage process to prevent yeast film formation.

Table 1. Inhibitory effect of GO, AA, and HG on film formation during cucumber pickle fermentation at 30°C for 30 days as compared with potassium sorbate

Concentrations (%)	Day of film appearance			
	GO[1)]	AA	HG	PS
0	3	3	3	3
0.001	-[2)]	-	-	-
0.002	5	5	-	4
0.003	7	-	-	-
0.004	8	-	-	-
0.005	12	7	-	5
0.006	>30	-	-	-
0.007	-	-	-	-
0.008	-	-	-	7
0.009	-	-	-	-
0.01	-	17	-	17
0.02	-	>30	-	>30
0.03	-	-	-	-
0.5	-	-	5	-
0.8	-	-	7	-
1	-	-	15	-
2	-	-	20	-
3	-	-	>30	-

[1)]GO, garlic oil; AA, allyl alcohol; HG, heated garlic; PS, potassium sorbate.
[2)]-: not tested.

Effects of pH on the inhibitory activities of HG and PS against *H. anomala* Effect of pH on the antimicrobial activity of HG was compared with that of a conventional antifungal preservative, potassium sorbate. The antimicrobial effect of HG against *H. anomala* was not influenced by pH. The MIC values remained at 1% from pH 3 to 7 in YMPG broth (Fig. 1). When the antimicrobial activity of HG was tested in CJ, however, the antimicrobial potency of HG increased as the pH increased from 4 to 7 for reasons which are not clear. The

Table 2. Minimum inhibitory concentration (MIC) of GO, HG, and AA against lactic acid bacteria[2] and yeasts[3] in different media

Microorganisms	MIC in CJ (%)			MIC in broths[4] (%)		
	GO[1]	HG	AA	GO	HG	AA
Lactobacillus plantarum LA 97	>0.1	>50	4	>0.1	>50	5
Lactobacillus plantarum ATCC 14917	>0.1	>50	5	>0.1	>50	4
Streptococcus feacalis	>0.1	>50	5	>0.1	>50	4
Pediococcus pentosaceus	>0.1	>50	4	>0.1	>50	5
Lactobacillus brevis KFCC 35464	>0.1	>50	4	>0.1	>50	2
Leuconostoc mensenteroides ATCC 8293	>0.1	>50	2	>0.1	>50	2
Leuconostoc dextranicum ATCC 19255	>0.1	>50	1	>0.1	>50	2
Hansenula anomala	0.008	2.5	0.0150	0.0020	1.0	0.0060
Pichia membranefaciens Y20	0.002	1.0	0.0010	0.0004	0.2	0.0002
Zygosaccharomyces rouxii KCCM 50523	0.008	2.5	0.0040	0.0010	1.0	0.0020
Candida utilis ATCC 42416	0.006	1.5	0.0015	0.0010	0.5	0.0010
Candida albicans KCTC 7965	0.012	2.0	0.0025	0.0040	1.5	0.0020
Saccharomyces cerevisiae ATCC4126	0.008	2.5	0.0050	0.0005	1.0	0.0010

[1]GO, garlic oil; HG, heated garlic; AA, allyl alcohol; CJ, cucumber juice.
[2]MIC (minimum inhibitory concentration) after 24 hr of incubation.
[3]MIC after 48 hr of incubation.
[4]Broths with 3.6% NaCl: MRS broth for lactic acid bacteria and YMPG broth for yeasts.

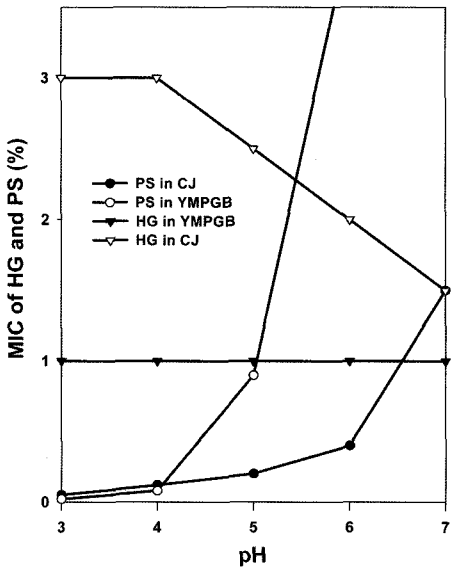

Fig. 1. Minimum inhibitory concentrations (MIC) of HG and PS against *Hansenula anomala* at different pH in different media.

antimicrobial activity of PS is very dependent on pH in both media as has been known for a long time (10). Natural antimicrobial materials such as GO, HG, and AA derived from garlic showed strong inhibitory activities against yeasts including film-forming yeasts (*H. anomala*, *P. membranefaciens* Y20, and *Z. rouxii* KCCM 50523), while having little effect on the growth of LABs. GO, HG, and AA were effective in preventing film formation in cucumber pickles for up to 30 days at concentrations of 0.006, 3, and 0.02%, respectively, without interfering with normal lactic acid fermentation. The anti-yeast activity of HG in YMPG broth was not influenced by pH, while that of PS was highly dependent on pH. Interestingly, the antiyeast activity of HG against *H. anomala*, when tested in CJ, increased with pH from 4 to 7.

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