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Changes in Microorganisms, Enzyme Activities, and Gas Formation by the Addition of Mustard Powder on *Kochujang* with Different Salt Concentration

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Abstract *Kochujang* was fermented using hot red pepper, *meju* prepared with soybean and rice, and malt-digested syrup. To reduce salt content, mustard powder (1.2%, w/w) was added to Korean traditional *kochujang* with 4-10% salt, and microbial characteristics, enzyme activities, and gas formation in *kochujang* were evaluated during fermentation for 120 days at 25°C. Yeast numbers of all treatments maintained 2.43-2.86 log CFU/g up to 60 days fermentation, indicating salt concentration had no effect on yeast count. Activities of α- and β-amylases, and neutral and acidic proteases of *kochujang* added with mustard powder were slightly higher than those of control group. Total accumulative volume of gas produced during fermentation of *kochujang* without mustard powder (control group) was 5,892 mL/pack, but decreased to 34-99 mL/pack in low-salted *kochujang* (4 and 6% salt) added with mustard powder. Major gas produced was carbon dioxide (79-80%) with oxygen content less than 1.25%(v/v). Results indicate salt concentration of *kochujang* could be lowered up to 6-8% by addition of mustard powder without gas formation and quality alteration during distribution.

Keywords: *kochujang*, mustard, gas formation, low-salt, yeast

Introduction

Kochujang is a traditional condiment in Korea that combines a sweet taste from a starch hydrolyzate, hot taste of red pepper, and savory taste from the soybean protein hydrolyzate and nucleic acids. It uses soybean and red pepper powder as main ingredients mixed with malt-digested rice, barley, or sweet potatoes according to the manufacturing region, and is divided into traditional *kochujang* made with *meju*, a kind of *koji*, and mass-produced *kochujang* made with *Aspergillus oryzae*; currently, traditional *kochujang* is mass-produced only in the Sunchang region (Korea), and the product quality decreases during distribution due to such factors as rising. The most prominent microorganisms present in *meju* of traditional *kochujang*, in which various microorganisms participate during fermentation, are bacteria, and the two major bacteria are *Bacillus subtilis* and *B. licheniformis*, both of which give special flavors to *meju* after long fermentation with the bacteria inside and fungi on the outer surfaces of the *meju* (1, 2). Mesophilic bacterial count during fermentation of *kochujang* ranges 7-8 log CFU/g (3, 4).

Korean and Japanese have taken a growing interest in *kochujang*, due to its effects of weight and blood-pressure reductions (5), and high poly-γ-glutamate content (6). However, the consumption of *kochujang* has certain dietary restrictions due to high salt concentration (about 15.01±6.48%), necessitating the reduction of salt concentration in *kochujang* (7, 8).

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Unfortunately, low salinity in *kochujang* decreases the preservation, and results in abnormal fermentation such as oxidation and gas formation by yeasts such as *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* during the fermentation process (9, 10). In previous studies, we confirmed the possibility of fermentation control in *kochujang* by the addition of mustard powder as a natural preservative (11, 12). Growth of the yeasts was reported to be delayed by allyl isothiocyanate (AITC) contained in spices such as horseradish and mustard (13, 14), and capsaicin (15). In particular, water extract (16) and distilled components (17) of mustard seeds, and horseradish and mustard (18) were reported to exhibit significant antimicrobial activities against bacteria and yeasts causing food poisoning, and essential oil of mustard seeds was reported to have minimum inhibitory concentration of 20-50 μL/petri-dish against food poisoning bacteria such as *B. cereus* (19).

In this study, to restrict gas formation by yeast during distribution, mustard powder was added to *kochujangs* with different salt concentrations, and changes in microorganisms, enzyme activities, and gas formation during fermentation of *kochujang* added with mustard powder were evaluated.

Materials and Methods

Materials A Dabok variety of red peppers and a Baektae variety of soybeans produced in the autumn, 2004 (Sunchang, Chonbuk, Korea) were used. Glutinous rice, product of Donggye, Sunchang, and domestic sun-dried salts were used for *kochujang* preparation. Mustard powder was obtained from Ottogi Co. (Seoul, Korea). Mustard seeds from Canada were defatted using an

expeller (residual oil 13-14%) and were ground into powder in three steps (8, 40, and 120 mesh).

***Kochujang* preparation** The mixing ratio for preparation of *kochujang* according to the traditional methods of Sunchang area (Chonbuk, Korea) is given in Table 1. Glutinous rice was soaked overnight, ground, mixed with malt extract, saccharified for 1 hr at 60°C, and filtered. *Kochujangs* were divided into five groups depending on the addition of salt and mustard powder during production. That is, control (Sunchang traditional *kochujang* without mustard powder) was made with 10% salt, and treated groups were added with 4, 6, 8, and 10% (w/w) salts and 1.2% (w/w) mustard powder containing 314 ppm AITC (11). One hundred fifty grams of each sample was filled in packing film (150×200 mm, nylon/15 μm + polyethylene/15 μm + linear low density polyethylene/60 μm; Sungil Chemicals Co., Chungju, Korea) and sealed using a sealer (Samyang Co., Seoul, Korea). *Kochujangs* were fermented for 120 days at 25°C in an incubator and were analyzed at 30-day intervals.

Viable cell counts Five grams of *kochujang* were serially diluted tenfold with 0.1% peptone water, and were dispersed on solidified plate count agar (Difco, Detroit, MI, USA) for bacteria and Yeast & Mold Count Plates (petrifilm™; 3M, St. Paul, MN, USA) for yeast. Bacteria were incubated at 30°C for 24 hr, and yeast at 25°C for 4-5 days, and were counted.

Amylase and protease activities The extracts of *kochujang* (10 g each) were prepared by shaking in 100 mL distilled water for 4 hr at room temperature and centrifugation at 17,000×g for 10 min (Model J2-21; Beckman Instruments, Inc., Palo Alto, CA, USA). The supernatant (as extract) was then gathered for determination of amylase and protease activities (20).

To measure α-amylase activity, 1 mL supernatant was added to a mixture of 1 mL of 1% soluble starch (pH 5.0) and 1 mL of acetate buffer, and heated at 40°C for 30 min. Ten mL of 0.5 M acetic acid was then added to stop the reaction, after which 10 mL of 3.33×10^{-4} N iodine solution was added. The activity of the reaction mixture was measured at 700 nm using a UV-spectrophotometer (UV-1201; Shimadzu Co., Kyoto, Japan) and expressed as per mL supernatant.

β-Amylase activity was determined by the dinitrosalicylic acid method. Briefly, a mixture of 1 mL of 0.5% soluble starch and 1 mL acetate buffer was added to 1 mL supernatant and reacted at 30°C for 30 min. One unit of β-amylase activity was expressed as the liberation of 1 mg maltose per mL supernatant.

Aliquots of 1 mL supernatant were used to measure the protease activity; pH was adjusted to 3.0 (acidic) or 6.0 (neutral). Five milliliters of 0.6% casein was added and heated at 30°C for 30 min. The reaction was stopped by adding 5 mL of 0.4 M trichloroacetic acid, and filtered. To 2 mL reaction mixture, 5 mL of 0.55 M Na_2CO_3 and 1 mL diluted Folin reagent were added. The protease activity was measured at 660 nm by a UV-spectrophotometer, and one unit of the activity was expressed as the liberation of 1 μg tyrosine per g *kochujang*.

Gas formation and ratio of CO_2-O_2 Gas was withdrawn from packing using a disposable medical syringe (50 mL) through silicon applied on to the surface of plastic film. Total gas formation was expressed as a cumulative value at 8, 10, 12, 13, and 120 days of fermentation. The ratio of carbon dioxide and oxygen in gas was determined using a CO_2-O_2 analyzer (Abiss VAK-12, Viry Chatillon, France).

Table 1. The mixing ratio of raw ingredients for the preparation of *kochujang*

Raw materials	Treatments (%, w/w)	
	Control	Mustard powder
Glutinous rice	22.2	22.2
Red pepper powder	23.1	23.1
Meju powder	6.2	6.2
Malt-digested syrup	38.5	43.3-37.3
Salt	10.0	4, 6, 8, 10
Mustard powder	0	1.2
Total	100	100

Results and Discussion

Changes in viable cell count of bacteria and yeast Changes in viable bacterial counts during fermentation of *kochujang* made with mustard powder and varying salt concentration are presented in Fig. 1. Bacterial counts of all treatments slightly increased up to 60-days of fermentation, and maintained a level of 8.34-8.76 log CFU/g during fermentation. No significant differences were observed among the treatments, indicating viable cell count of bacteria in *kochujang* was not affected by the addition of mustard powder. The results of this study were consistent with those of Shin *et al.* (11), who reported that the viable cell count of *kochujang* was unaffected by the addition of horseradish or mustard, and those of others, who found the viable cell counts of traditional *kochujang* collected from various regions in Korea were 10^7-10^8 CFU/g (7, 21). *Kochujang*, which contains a high amount of reducing sugar at 27.52±7.32%, pH 4.60±0.23, and 0.79

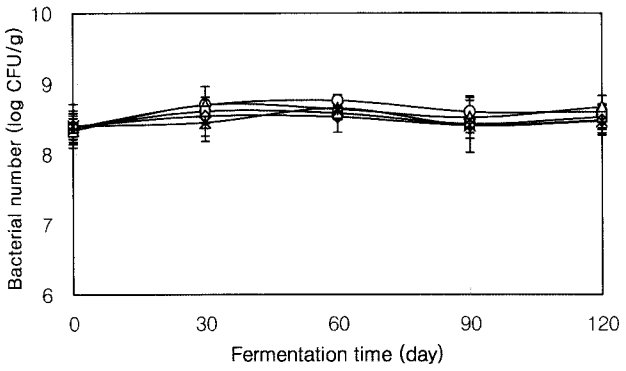

Fig. 1. Changes in bacterial number of *kochujang* added with mustard powder during fermentation at 25°C. ○, Control (salt 10%); △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%; ◇, Salt 8% + mustard 1.2%; ×, Salt 10% + mustard 1.2%. Vertical bars represent standard deviation (n=3).

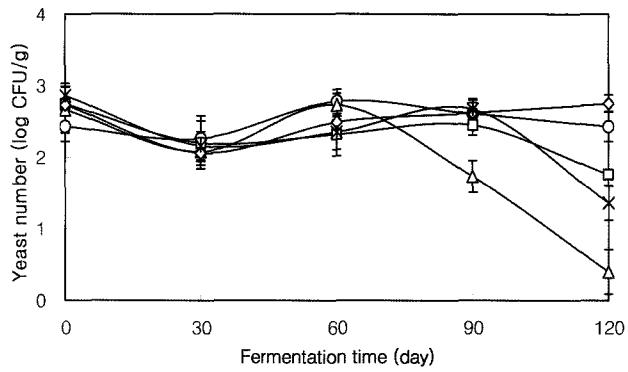

Fig. 2. Changes in yeast number of *kochujang* added with mustard powder during fermentation at 25°C. ○, Control (salt 10%); △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%; ◇, Salt 8% + mustard 1.2%; ×, Salt 10% + mustard 1.2%. Vertical bars represent standard deviation (n=3).

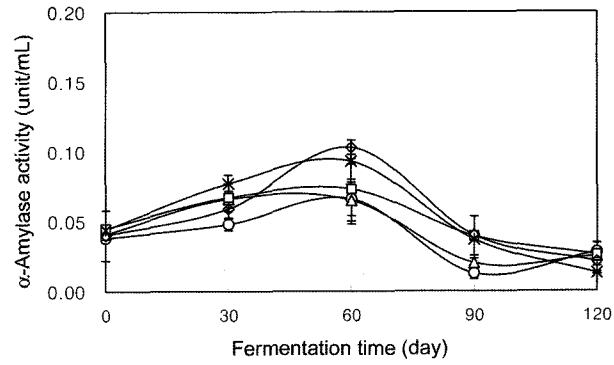

Fig. 3. Changes in α-amylase activity of *kochujang* added with mustard powder during fermentation at 25°C. ○, Control (salt 10%); △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%; ◇, Salt 8% + mustard 1.2%; ×, Salt 10% + mustard 1.2%. Vertical bars represent standard deviation (n=3).

±0.04 water activity, provides unfavorable condition for the growth of microorganisms (7); thus, the microorganisms in it remain in static state. In the present study, no difference was observed in the viable cell counts of *kochujang* by salt concentration, because *kochujang* had low water activity. Shofran *et al.* (13) reported that AITC, which is a major antimicrobial constituent of Cruciferae plants such as mustard, had a minimum inhibitory concentration (MIC) of 50 to 1,000 ppm for bacteria. We assumed that the viable bacteria counts of *kochujang* were unaffected due to the addition of mustard powder at a low level (3.7 ppm AITC).

Changes in yeast numbers during fermentation of *kochujang* added with mustard powder depending on salt concentration are shown in Fig. 2. Yeast counts of all treatments were maintained at the range of 2.43-2.86 log CFU/g up to 60 days fermentation. However, at 120 days fermentation the yeast numbers of all treatments except for the control (2.43 log CFU/g) and the 8% salt treatment (2.75 log CFU/g) decreased to 0.40-1.76 log CFU/g, consistent with the results showing that the yeast numbers of low-salted *kochujang* added with horseradish powder (3) and mustard powder (22) decreased at later period of fermentation. Shofran *et al.* (13) reported that AITC had MIC of 1 to 4 ppm for nonxerotolerant yeasts, whereas, against xerotolerant yeasts at 50 ppm, it retarded but did not prevent the growth. Although the mechanisms by which AITC inhibit microorganism activity are not well-known, various hypothesis including oxidative cleavage of disulfide bond (23), inhibition of oxygen uptake (25), and electrophilic attack on cysteine (24) have been proposed. Results of our study show that the addition of mustard powder had little effect on the yeast numbers of *kochujang*, possibly because traditional *kochujang* has both nonxerotolerant and xerotolerant yeasts (*Z.* and *S. rouxii*) (10). In addition, the yeast numbers of low-salted *kochujang* showed no correlation with the salt concentration.

Changes in amylase activities Changes in α-amylase activities during fermentation of *kochujang* added with mustard powder depending on salt concentration are shown in Fig. 3. α-Amylase activities of all treatments increased up to 60 days fermentation, and decreased thereafter. After 60 days fermentation, α-amylase activities of 8 and 10% salt treatments were slightly higher than those of other groups, although no significant difference was observed depending on the salt concentration. Our results were consistent with those of Kim *et al.* (26), who reported that the enzymes in *kochujang* showed maximum activity at 40-50 days fermentation and decreased thereafter. Oh and Park (27) reported that the enzyme activities in *kochujang* using aged *meju* reached maximum at 30 days fermentation and decreased thereafter.

β-Amylase activities of *kochujang* added with mustard powder showed an increasing tendency during fermentation (Fig. 4). However, at 30 days fermentation β-amylase activities of the control group were lower (1.56-1.93 unit/mL) than those of other treatments (2.59-3.76 unit/mL), similar to those of Shin *et al.* (11), who reported that the enzyme activities of treatments added with horseradish and mustard were higher (1.276-1.918 unit/g) than those of other treatments. However, these results differed from those of other group showing that β-amylase activities of *kochujang* added with alcohol, mustard powder, or chitosan increased up to mid fermentation, but at slightly lower level than those of the control group (22).

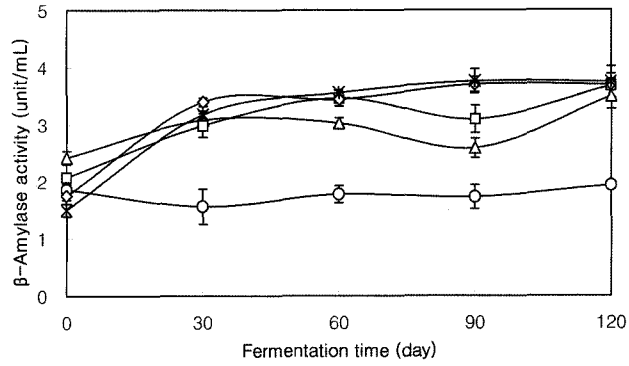

Fig. 4. Changes in β-amylase activity of *kochujang* added with mustard powder during fermentation at 25°C. ○, Control (salt 10%), △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%; ◇, Salt 8% + mustard 1.2%; ×; Salt 10% + mustard 1.2%. Vertical bars represent standard deviation (n=3).

Activities of α- and β-amylases of *kochujang* added with mustard powder were higher than those of the control group during fermentation, with no consistent correlation with salt concentration.

Changes in protease activities Neutral protease activities of *kochujang* added with mustard powder gradually increased during fermentation and showed highest activities in all treatments at 120 days fermentation (Fig. 5). During fermentation neutral protease activities of *kochujangs* added with mustard powder were higher than those of control group, different from those of Kim (22) and Kim *et al.* (28), who reported that neutral protease activity of *kochujang* increased up to mid fermentation and decreased thereafter.

Acidic protease activity showed trend similar to that of neutral protease during fermentation, and no significant difference in the treatments was observed except for the control group. These results were similar to those determined in *kochujang* added with horseradish and mustard (11) and *sikhae kochujang* (4).

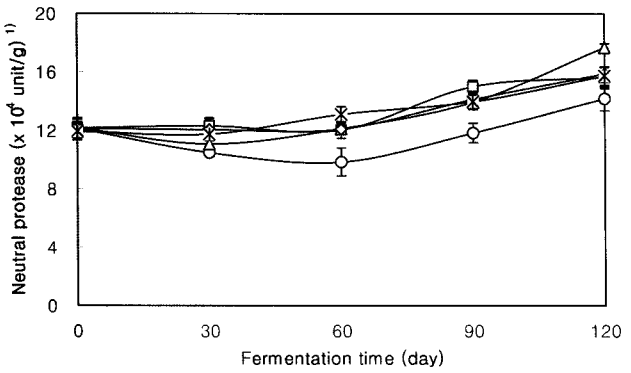

Fig. 5. Changes in neutral protease activity of *kochujang* added with mustard powder during fermentation at 25°C. ○, Control (salt 10%); △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%; ◇, Salt 8% + mustard 1.2%; ×, Salt 10% + mustard 1.2%. Vertical bars represent standard deviation (n=3). [1]Basis on dry weight.

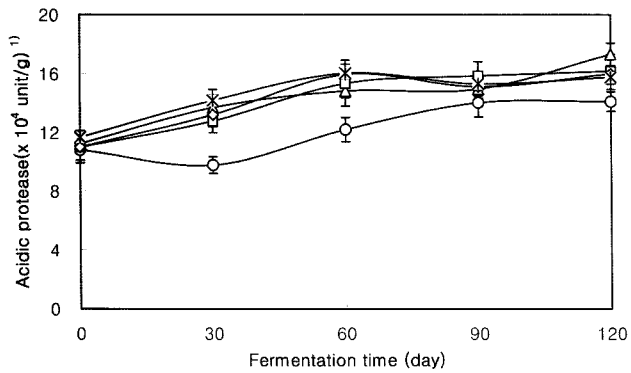

Fig. 6. Changes in acidic protease activity of *kochujang* added with mustard powder during fermentation at 25°C. ○, Control (salt 10%); △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%; ◇, Salt 8% + mustard 1.2%; ×, Salt 10% + mustard 1.2%. Vertical bars represent standard deviation (n=3). [1] Basis on dry weight.

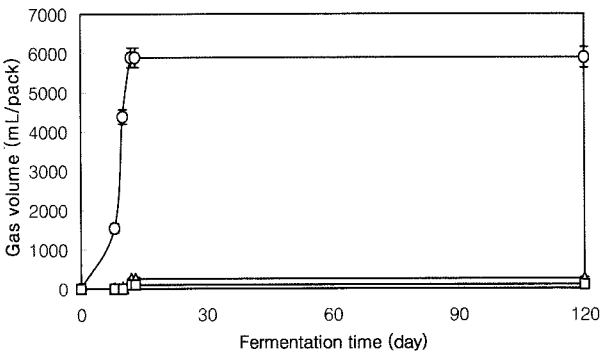

Fig. 7. Total accumulative volume of gas produced in *kochujang* pack added with mustard powder during fermentation at 25 °C. ○, Control (salt 10%); △, Salt 4% + mustard 1.2%; □, Salt 6% + mustard 1.2%. Vertical bars represent standard deviation (n= 3).

Activities of neutral and acidic proteases in *kochujang* added with mustard powder were slightly higher than those of the control group during fermentation, with no significant difference observed depending on the salt concentration. In addition, the enzyme activities of *kochujang* were unaffected by the addition of mustard powder.

Gas formation and its composition Accumulative gas volumes formed by yeast during fermentation of *kochujang* are shown in Fig. 7. In the control group, significant amount of gas was formed (5,892 mL/pack) up to 12 days fermentation, then maintained a constant rate thereafter. However, gas formation was not observed up to 8 days fermentation in 4% salt treatment, whereas at 12 days fermentation 34 mL/pack gas was formed. In addition, in the case of 6% salt treatment, gas was not formed up to 10 days fermentation, then formed 99 mL/pack at 12 days fermentation. The major gas produced in *kochujang* during fermentation was carbon dioxide (79-80%, v/v), with less than 1.25% (v/v) oxygen content (Fig. 8).

These results show addition of mustard powder to low-salted *kochujang* had no effect (α-amylase) or only a slight

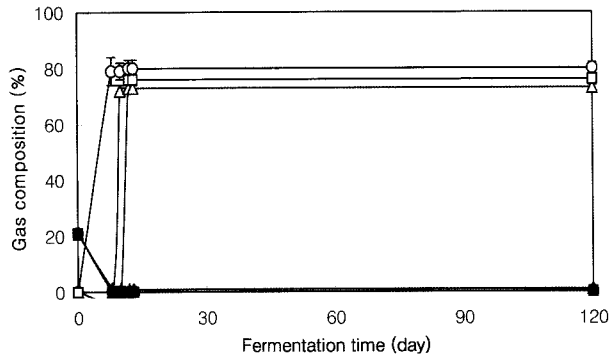

Fig. 8. Gas composition of *kochujang* pack added with mustard powder during fermentation at 25°C. ○, CO_2 (salt 10 %); △, CO_2 (salt 4% + mustard 1.2%); □, CO_2 (salt 6% + mustard 1.2 %); ●, O_2 (salt 10%); ▲, O_2 (salt 4% + mustard 1.2%); ■, O_2 (salt 6% + mustard 1.2%). Vertical bars represent standard deviation (n=3).

increase (β-amylase and protease) in enzyme activities. Mustard powder probably had no effect on yeast number (Fig. 2), but inhibited the gas formation by yeast during fermentation. We previously determined that the taste and overall acceptability of *kochujang* (>6% salt) added with mustard powder scored higher than the control group (29). Based on these findings, we established that the concentration of salt (10%) added to *kochujang* could be lowered up to 6-8% by the addition of mustard powder (1.2%, w/w), which also prevented gas formation during distribution, without affecting the quality of *kochujang*.

Acknowledgments

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