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Inhibitory Activity of Garlic Fermented by *Pediococcus pentosaceus* KACC 91419 against Antibiotic-resistant Pathogens

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ABSTRACT : The aim of this study was to screen lactic acid bacteria for the fermentation of garlic and to assess the increase in inhibitory activity of garlic fermented against antibiotic-resistant pathogens for use as an animal feed supplement. We screened 45 strains of lactobacillus for the fermentation of garlic. Of these strains, 23 showed similar growth rates with or without allicin. Cultures of the 23 strains were mixed with an equivalent amount of garlic juice and incubated overnight at 37°C. The three strains with the lowest pH values were *Lactobacillus paracasei* KCTC 3169, L5 strain, and *L. reuteri* SW. Garlic juice fermented by the L5 strain more strongly inhibited antibiotic-resistant pathogenic bacteria than *L. paracasei* KCTC 3169, *L. reuteri* SW, or garlic juice itself. By examining carbohydrate utilization, morphologic properties and 16S rRNA gene sequences, we identified the L5 strain as *Pediococcus pentosaceus* and deposited it in the name of *P. pentosaceus* KACC 91419 into the Korea Agricultural Culture Collection. To identify the antimicrobial compound from the garlic filtrate fermented by *P. pentosaceus* KACC 91419, we fractionated *P. pentosaceus* KACC 91419 culture on a C_{18} column and checked the antimicrobial activity of fractions A6 to A10. Only fraction A9 showed inhibitory activity on *Staphylococcus aureus*. Comparing the mass spectra of the fractions with and without antimicrobial activity, we observed a single dominant product ion (*m/z* 157.99) from the fraction showing antimicrobial activity. Its molecular mass (157.99) was 2 atomic mass units less than that of allicin (162.02). This suggests that allicin might be converted to its derivative, which has antimicrobial activity, during fermentation by *P. pentosaceus* KACC 91419. (**Key Words :** *Pediococcus pentosaceus* KACC 91419, Garlic Fermentation, Antibiotics Resistance)

INTRODUCTION

Garlic (*Allium sativum*) is used worldwide as a food additive and medicine. Garlic extract inhibits the growth of Gram-positive and Gram-negative bacteria, including *Micrococcus*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Proteus*, and *Helicobacter pylori* (Sivam et al., 1997; Ankri and Mirelman, 1999; Ross et al., 2001; Tsao and Yin, 2001; Martin and Ernst, 2003). Its antibacterial activity is mainly due to allicin, (+)-S-allyl-L-cysteine sulfoxide, produced by the enzymatic activity of allinase (a cysteine sulfoxide lyase) on alliin, after the clove is crushed or cut (Ellmore and Feldberg, 1994; Ross et al., 2001). Garlic is even active against microorganisms that are resistant to antibiotics (Jezowa et al., 1966), and the combination of garlic extracts with antibiotics leads to partial and total synergism (Didry et al., 1992).

Many types of antibiotics are used in the animal industry. Antibiotics improve feed conversion and body weight gain presumably by altering the composition and activities of microflora (Knarreborg et al., 2002; Collier et al., 2003). Adding antibiotics to animal feeds may modify the intestinal flora and create selective pressure in favor of resistant bacteria (Aarestrup, 2000; Singer and Hofacre, 2006). Resistance may be inherent, which explains the phenomenon of opportunistic infection or acquired immunity. In the late 1990s, concerns about drug resistance increased, and since then, numerous reports have implicated improper agricultural use as the main culprit, advising tighter controls, more appropriate choices and regimens, prevention of cross-infection, and development of new microbial substances. The emergence of multi-drug-

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resistant strains of Gram-negative (*Pseudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Salmonella* spp., etc.) and Gram-positive (*Staphylococcus*, *Enterococcus*, *Streptococcus* spp., etc.) bacteria is troubling for human and animals (Sharma et al., 2005). In response to the emergence of resistance to antibiotics, European countries have restricted or banned the use of antibiotics as growth promoters (Singer and Hofacre, 2006).

Therefore, many alternatives to antibiotics, such as probiotics, organic acids, natural herbs, and so on, are being developed for use in the animal industry. Garlic may be one promising alternative. Chickens infected with *Candida albicans* were successfully treated with garlic (Prasad and Sharma, 1980). However, little is known about whether garlic fermented by lactic acid bacteria increases the inhibition of antibiotic-resistant bacteria. The aim of this study was to screen lactic acid bacteria for the fermentation of garlic and to assess the increase in the inhibitory activity of garlic fermented against antibiotic-resistant pathogens for use as an animal feed supplement.

MATERIALS AND METHODS

Screening of lactic acid bacteria for the fermentation of garlic

The National Institute of Animal Science (Suwon, Korea) supplied 45 strains of lactobacillus previously obtained from Korean Collection for Type Cultures (Daejeon, Korea) or isolated from silages and dairy products. The strains were grown in MRS broth (Difco, USA) with and without 5 mg/ml allicin (Tianjin Haishengweibang Fine Chemical Co., China), and lactobacilli showing more than 90% growth in the presence of allicin were selected for study. Cell growth was checked by measuring optical density at 600 nm. Garlic juice was prepared by centrifugation at 12,000×*g* for 20 min. after crushing garlic, and MRS cultures of the selected strains were each mixed with the same amount of garlic juice. After incubating overnight at 37°C, the strain associated with a lower pH value than the garlic and higher antimicrobial activity was selected for further study.

Identification and characterization of lactic acid bacteria

The cell morphology of the selected strain was examined using scanning electron microscopy (XL30CP, Philips). The colony on MRS agar (Difco, USA) was picked and transferred to filter paper, and fixed with 8% paraformaldehyde and 3% glutaraldehyde in cacodylate buffer overnight. After washing and dehydration, sample was coated with gold and observed under scanning electron microscopy.

Its carbohydrate utilization pattern was further characterized using the API-50 CH system (bioMerieux, France).

To identify the lactic acid bacteria isolate, we performed 16S rRNA gene sequence analysis according to the method of Pavlova et al. (2002). To amplify 16S rDNA, we used universal primers corresponding to six conserved regions of the *Escherichia coli* numbering system. Chromosomal DNA was isolated using a genomic DNA extraction kit (Qiagen, Germany). Polymerase chain reaction was performed in a 50 μl reaction mixture containing primers (50 pmol), template DNA (50 ng), 5 μl 10×*Taq* DNA polymerase buffer, 4 μl dNTP at 2.5 mM, and 1 U *Taq* DNA polymerase (Takara, Japan). The polymerase chain reaction amplification product was purified using a QIAquick gel extraction kit (Qiagen), ligated into a pSTBlue-1 vector (Novagen, USA), and transformed into *E. coli* DH5α competent cells. The recombinant plasmids were purified using a DNA purification kit (Qiagen) and digested with *Eco*RI to confirm the insert. The nucleotide sequence of the insert was determined using a BigDye™-terminator sequencing kit and ABI PRISM 377 sequencer (Perkin-Elmer, USA), according to the manufacturer's instructions. The 16S rDNA sequences were subjected to a similarity search of the GenBank database.

Antimicrobial activity assay of fermented garlic juice

An agar-well diffusion method modified from Tagg and McGiven (1971) was used. In brief, Petri dishes containing sterile metal borers 8 mm in diameter were filled with Tryptic Soy Agar (Difco) and inoculated with the test strains. After solidification, the borers were removed and the base of each hole sealed with a drop of melted Tryptic Soy Agar. Then, 50 μl of each fermented garlic culture was added to the wells. The inoculated plates were incubated at 37°C for 1 day after an overnight pre-incubation at 4°C.

Test strains for the antimicrobial activity assay

Staphylococcus aureus KCCM 40510 (methicillin-resistant) (Lee et al., 2005), *E. coli* F4 (Ham et al., 2003), and *Salmonella typhimurium* BAA-185 (American Type Culture Collection, USA) were grown overnight at 37°C in Tryptic Soy Broth (Difco). Susceptibility of the test strains to antibiotics was examined using Antibiotics Disc (Becton, Dickinson and Company, USA).

Identification of a compound with antimicrobial activity from fermented garlic

Garlic cultures fermented by the selected lactic acid bacteria were filtered by passing through a 10 kDa molecular weight cut-off membrane (Millipore, USA). The filtrates were tested for inhibitory activity against *S. aureus* KCTC 40510. After confirmation of antimicrobial activity, the filtrate was fractionated on a C_{18} column (Fisher

Table 1. Variation in the growth rate of 45 lactobacilli with the addition of allicin (5 mg/ml)

Growth rate	<80	80-90	90-100	100<
Number of strains	12	10	16	7

Scientific, USA) with the mixture of methanol and deionized water (5:95 v/v) as the mobile phase and fractions collected to check for antimicrobial activity. Fractions with antimicrobial activity were subjected to liquid chromatography-mass spectrometry (LC-MS) to compare the MS spectrum of each fraction.

LC-MS analysis

LC-MS analysis was performed using an integrated system consisting of a nano pump, an auto-sampler (Tempo nano LC system; MDS SCIEX, Canada) and a hybrid Quadrupole-TOF MS spectrometer (Qstar Elite; Applied Biosystems, USA) equipped with a nano-electrospray ionization source. Each fraction was reconstructed in solvent A (water:acetonitrile (98:2 v/v), 0.1% formic acid) and then separated on a Zorbax 300SB-C_{18} capillary column (85 μm i.d.×160 mm, 3.5 μm, 300A; Agilent Technologies, USA) at a flow rate of 300 nL/min. The LC gradient was run at 2% to 35% solvent B (water:acetonitrile (2:98 v/v), 0.1% formic acid) over 20 min, then from 35% to 90% over 15 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Resulting fractions were electrosprayed at an ion spray voltage of 2,300 eV. Mass data were acquired automatically using Analyst QS 2.0 software (Applied Biosystems).

RESULTS

Screening of lactic acid bacteria for the fermentation of garlic

Table 1 shows the growth variation among lactic acid bacteria in MRS broth with and without allicin. Of the 45 strains of lactic acid bacteria, 16 displayed similar growth irrespective of allicin and 7 strains showed improved growth with the addition of allicin. Although garlic preparations exhibit a broad spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria, other bacterial strains such as the mucoid strains of *Pseudomonas aeruginosa*, *Streptococcus* β-*hemolyticus* and *Enterococcus faecium* were resistant to allicin (Ankri and Mirelman, 1999). It was assumed that hydrophilic capsular or mucoid layers prevent the penetration of allicin into the bacteria; this should be confirmed (Ankri and Mirelman, 1999). Cho et al. (2001) reported that some lactobacilli may display improved growth with allicin.

Cultures of the 23 strains showing more than 90% growth in the presence allicin were mixed with the same amount of garlic paste and pH values measured after the strains incubated overnight at 37°C. The three cultures strains with the lowest pH values after incubation as shown in Table 2 were selected. Whereas the pH of the control (MRS plus garlic paste) was hardly changed during incubation, those of the garlic paste fermented with *Lactobacillus paracasei* KCTC 3169, L5 strain, and *L. reuteri* SW decreased to 4.56, 4.70, and 4.79, respectively. This suggests that these three strains can grow in the medium containing garlic paste.

Table 2. pH change in lactobacilli culture mixed with an equivalent amount of garlic paste after overnight incubation

Lactic acid bacteria	pH	
	Before incubation	After incubation
Control (MRS+garlic paste)	6.65	6.71
L. paracasei KCTC 3169	4.70	4.56
L5 strain	4.93	4.70
L. reuteri SW	4.92	4.79

Resistance of the indicator strains to antibiotics

The resistance of four pathogenic bacteria to antibiotics was tested. As shown in Table 3, *E. coli* F4 showed resistance against kanamycin, penicillin, tetracycline, and vancomycin. *S. typhimurium* BBA 185 showed resistance against chloramphenicol, penicillin, streptomycin, and vancomycin. *S. aureus* KCTC 40510 showed resistance against erythromycin, penicillin, and streptomycin. All of the strains tested showed resistance against penicillin.

Antimicrobial activity of fermented garlic

The antimicrobial activity of the garlic fermented with the selected lactic acid bacteria was checked by measuring the clear zone diameter in the agar plate overlayed with the indicator strains. As shown in Table 4, the fermented garlic paste displayed stronger inhibitory activity against all indicator strains than garlic paste itself. Inhibitory activity varied by strain. Overall, garlic fermented by the L5 strain

Table 3. Profiles of test strain susceptibility to antibiotics

Strains / Antibiotics	*E. coli* F4	*S. typhimurium* BBA 185	*S. aureus* KCTC 40510
Chloramphenicol	S	R	S
Erythromycin	S	S	R
Kanamycin	R	S	S
Penicillin	R	R	R
Streptomycin	S	R	R
Tetracycline	R	S	S
Vancomycin	R	R	S

* R = Resistant; S = Susceptible.

* Antibiotics disc: chloramphenicol 30 μg, erythromycin 15 μg, kanamycin 30 μg, penicillin 10 IU, streptomycin 10 μg, tetracycline 30 μg, vancomycin 30 μg.

Table 4. Antimicrobial activity of garlic fermented with selected lactic acid bacteria

LAB / Test strain	*E. coli* F4	*S. typhimurium* BBA 185	*S. aureus* KCTC 40510
Garlic	+	+	+
L5 strain	+++	++	+++
L. paracasei KCTC 3169	++	++	++
L. reuteri SW	++	++	++

* + ~ +++: Iinhibition degree.

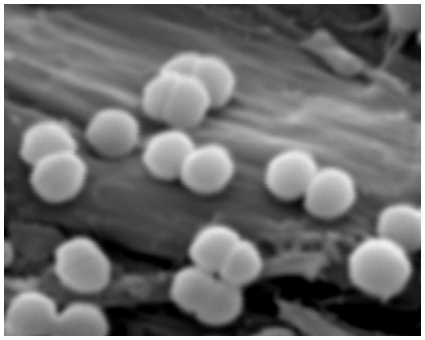

Figure 1. Scanning electron micrograph of the L5 strain (bar = 1 μm).

more strongly inhibited antibiotic-resistant pathogenic bacteria than that fermented by *L. paracasei* KCTC 3169 or *L. reuteri* SW.

Identification and characterization of the L5 strain

The L5 strain, which most strongly inhibited pathogenic bacteria, was subjected to further study. It was found to be Gram-positive, non-motile, catalase-negative, and a non-spore-forming strain (data not shown). Under an electron microscope, the cells appeared to be spherical and form tetrads (Figure 1). The carbohydrate utilization pattern of the L5 strain is shown in Table 5. The L5 strain could utilize fructose, D-glucose, and mannose; but did not utilize sorbose, inulin, or inositol. Based upon Bergey's Manual (Kandler and Weiss, 1986) and the utilization of carbohydrates, we identified the L5 strain as *Pediococcus pentosaceus* (98.0%). The strain was further examined using 16S rDNA sequence analysis. To amplify full-length 16S rDNA, we used universal primers corresponding to six conserved regions of the *E. coli* numbering system (Pavlova et al., 2002). The 16S rDNA was amplified by polymerase chain reaction and subjected to a similarity search of the GenBank database. The 16S rRNA gene sequences (Figure 2) of the L5 strain matched perfectly those of *P. pentosaceus* ATCC 25745 (99.8%). Thus, the L5 strain was officially identified as *P. pentosaceus* and deposited in the name of *P. pentosaceus* KACC 91419 to Korea Agricultural Culture Collection (Suwon, Korea).

Isolation and characterization of the antimicrobial compound from *P. pentosaceus* KACC 91419

The garlic cultures fermented by *L. fermentum* KACC 91419, *L. paracasei* KCTC 3169, and *L. reuteri* SW were

Table 5. L5 utilization of carbohydrate substrates

Carbohydrate	Utili-zation	Carbohydrate	Utili-zation	Carbohydrate	Utili-zation
Glycerol	-	Mannitol	-	D-raffinose	+
Erythritol	-	Sorbitol	-	Amidon	-
D-arabinose	-	α-Methyl-D-Mannoside	-	Glycogen	-
L-arabinose	-	α-Methyl-D-Glucoside	-	Xylitol	-
Ribose	+	N acetyl glucosamin	-	β-Gentiobiose	-
D-xylose	-	Amygdalin	-	D-turanose	-
L-xylose	-	Arbutin	-	D-lyxose	-
Adonitol	-	Esculin	+	D-tagatose	-
β -Methyl-D-xyloside	-	Salicin	-	D-fucose	-
Galactose	-	Cellobiose	-	L-fucose	-
D-glucose	+	Maltose	+	D-arabitol	-
D-fructose	-	Lactose	-	L-Arabitol	-
D-mannose	-	Melibiose	+	Gluconate	-(+)
L-sorbose	-	Saccharose	+	2-keto-gluconate	-
Rhamnose	-	Trehalose	-	5-keto-gluconate	-
Dulcitol	-	Inulin	-		
Inositol	-	Melezitose	-		

+: positive, -: negative.

GGCGGGTGCCTATACATGCAAGTCGAACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGATTG
AGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGA
TAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGG
CTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGC
AGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAG
AAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTG
ACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGG
CTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTG
TAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGA
CGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATG
ATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAA

Figure 2. 16S rDNA partial sequences of the L5 strain.

filtered through a 10 kDa molecular weight cut-off membrane. All permeate showed inhibitory activity against *S. aureus* (Figure 3A), indicating that the antimicrobial compound has a molecular weight less than 10 kDa. Garlic filtrate fermented by *P. pentosaceus* KACC 91419 more strongly inhibited *S. aureus* than that fermented by other lactic acid bacteria strains. To identify the antimicrobial compound from the garlic filtrate fermented by *P. pentosaceus* KACC 91419, we fractionated the filtrate on a C_{18} column and checked fractions A6 to A10 for antimicrobial activity. Only fraction A9 had an inhibitory effect on *S. aureus* (Figure 3B).

LC-MS was performed on each fraction (A8 to A10) and the mass spectra were compared. Figure 4 compares the mass spectra for the fractions with (A9) and without (A8 and A10) antimicrobial activity. It is interesting that a single dominant product ion (*m/z* 157.99) was observed only from the fraction (A9) that showed antimicrobial activity. The molecular mass (157.99) was 2 atomic mass units less than that of allicin (162.02). This suggests that allicin might be converted to its derivative, which has antimicrobial activity, during fermentation by lactic acid bacteria. The proposed chemical structure of the allicin derivative that has an atomic mass of 157.99 is depicted in Figure 5. More work is needed to determine how derivative is formed, but it could be generated by dehydration of both sides of the propenyl group of allicin.

DISCUSSION

Garlic played an important dietary and medicinal role for centuries. Garlic contains characteristic organosulfur

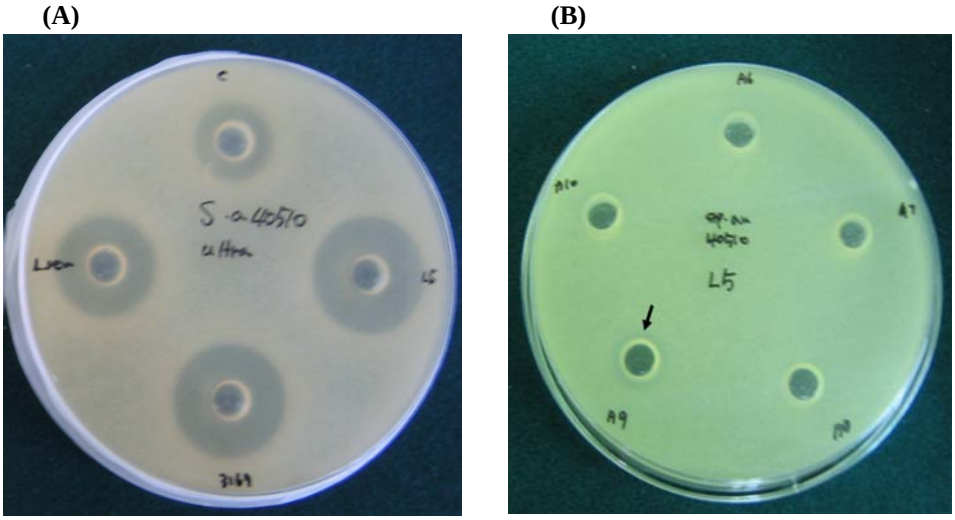

Figure 3. Antimicrobial activity of fermented garlic (A) and fractions of garlic fermented by *P. pentosaceus* KACC 91419 (B). *Staphylococcus aureus* KCTC 40510 was used as the indicator strain. (A) Top, control; bottom, garlic fermented by *L. paracasei* KCTC 3169; left, garlic fermented by *L. reuteri* SW; right, garlic fermented by *P. pentosaceus* KACC 91419. (B) Garlic juice fermented by *P. pentosaceus* KACC 91419 was filtered and fractionated from A6 to A10. The arrow in fraction A9 points to a clear zone, which indicates antimicrobial activity.

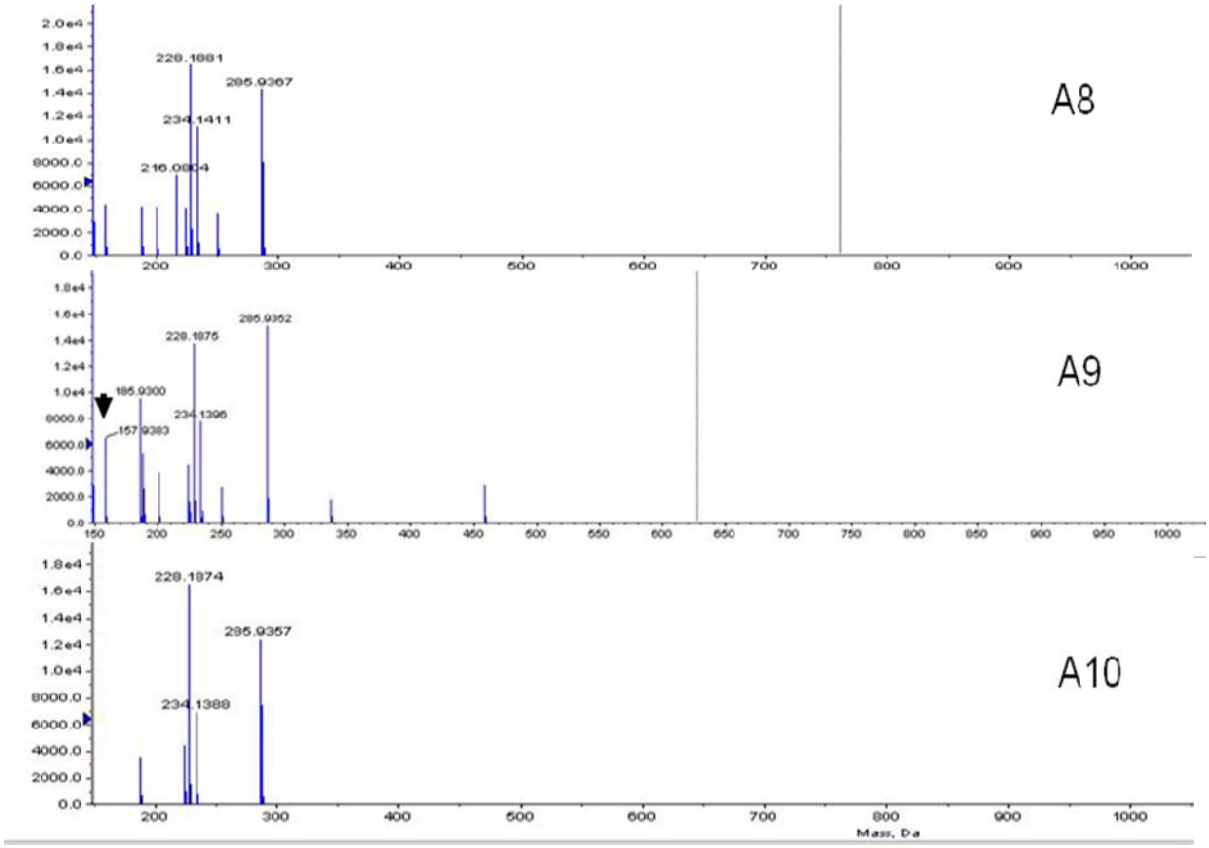

Figure 4. Mass spectra of fractions A8, A9, and A10 of garlic fermented by *P. pentosaceus* KACC 91419. The vertical scales reflect relative abundances. The arrow in fraction A9 indicates the new compound (*m/z* 157.99) derived from allicin.

compounds that contribute to its antimicrobial and pharmacologic activities (Yoshida et al., 1985; Singh et al., 1996). These organosulfur compounds are converted to other compounds during processing, which triggers the formation of a cascade of new compounds. Different processing techniques produce not only different compounds but also different constituents in the preparations (Amagase et al., 2001). Thus, different preparations of garlic may have different health benefits.

We isolated and characterized *P. pentosaceus* KACC 91419, which increases the antimicrobial activity of garlic juice. Compared with unprocessed garlic juice, garlic juice fermented by *P. pentosaceus* KACC 91419 strongly inhibits antibiotic-resistant *S. aureus*, *E. coli*, and *S. typhimurium*, but its inhibitory activity varies by strain. Garlic juice does not inhibit the growth of *P. pentosaceus* KACC 91419, even though allicin and its derivatives in garlic have bacteriostatic effects. Garlic exerts a differential inhibition between beneficial intestinal bacteria and potentially harmful enterobacteria (Rees et al., 1993). The structural differences of the bacterial strains may play a role in their susceptibility to garlic constituents (Tynecka and Gos, 1975). Furthermore increases in the antimicrobial activity of garlic by *P. pentosaceus* KACC 91419 is not only due to organic acids.

Although the garlic fermented by *L. paracasei* KCTC 3169 has a lower pH value than that fermented by the other strains, it does not show strong antimicrobial activity. This indicates that pH is not the most important factor of inhibitory activity and that some compounds that inhibit pathogenic bacteria are formed during the fermentation of garlic by *P. pentosaceus* KACC 91419. LC-MS analysis showed that a new compound is generated during the fermentation of garlic by *P. pentosaceus* KACC 91419. It is

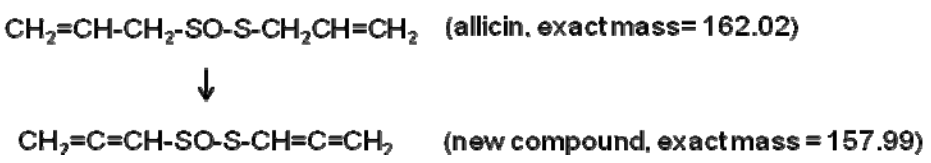

Figure 5. Proposed chemical structure of the compound derived from allicin.

hypothesized that allicin is converted to its derivative, which is 2 atomic mass units smaller, by dehydration of both sides of the propenyl group of allicin during fermentation. It is unknown if this derivative alone express antimicrobial activity or whether other compounds are required to produce a synergistic effect on the inhibition of pathogenic bacteria. Further fermentation experiments under various conditions using *P. pentosaceus* KACC 91419 or other lactic acid bacteria will help answer to these questions. The structure of the derivative appears to be rigid and reactive; thus, it is unstable during storage. Purification of the active compound and characterization of its mechanism of actions will be areas of future study.

The fermentation of herbal plants with lactic acid bacteria has not been well explored in the search for alternatives to antibiotics. Recently, Ko et al. (2008) reported that the addition of green tea fermented with several microorganisms to pig diet significantly reduced the feed conversion ratio in finishing pigs. Studies have yet to demonstrate that the fermentation of garlic by lactic acid bacteria increases garlic's antimicrobial activity. Microbial drug resistance is a difficult problem in the animal industry as well as the medical industry. More research is needed on the fermentation of garlic by lactic acid bacteria to assess the value of garlic as an alternative to antibiotics in the animal industry and biopreservatives for animal foods.

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