International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

A Lactic acid bacterium isolated from Korean fermented fish, Jeotgal: Characterization of *Weissella halotolerans* KNOUC4036 as a probiotic

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Abstract

This study was performed to isolate lactic acid bacteria (LAB) useful for probiotics from jeotgal, a Korean fermented fish food. Among five hundreds and ninety isolates taken from various jeotgal samples in Korea, we isolated 156 LAB, and selected a strain KNOUC4036 showing the highest antimicrobial activity. The selected strain was identified genotypically as *Weissella halotolerans*, and named as *Weissella halotolerans* KNOUC4036**.** Among 9.43log CFU/ml of the strain, 8.27 log CFU/ml survived after heating at 60℃ for 30 min, and 5.73 log CFU/ml among 8.41 log CFU/ml after treatment at pH 2 for 120 min at 37℃**.** Oxgall of 0.3% in MRS broth did not inhibit the growth of the strain. The strain showed the percentage of adhesion to organic solvents of hexadecane, toluene and xylene to be 79.96%, 69.51% and 60.37% respectively. Antimicrobial substance produced by the isolate was confirmed on SDS-PAGE gel by antimicrobial inhibitory zone against *Listeria monocytogenes*. The antimicrobial substance retained its antimicrobial activity at pH 2 to pH 8, and was stable at the treatment of 30℃ to 100℃ for 30 min and at 121℃ for 15 min. The antimicrobial substance lost its antimicrobial activity by proteolytic and amylolytic hydrolysis**.**

Keywords: LAB, jeotgal, antimicrobial activity, antimicrobial substance, probiotic, *Weissella halotolerans*

Introduction

Jeotgal is one of traditional fermented fish salty foods in Korea. It is made by adding 20 to 30% (w/w) salt to various types of seafood such as shrimp, oyster, shellfish, fish, fish eggs, and fish intestines and fermentation through subsequent preservation to palatable one (Guan et al., 2011). Aerobic and anaerobic bacteria, which are salt resistant, exist in most Jeotgal. It was reported that the fermentation of Jeotgal was largely performed by *Bacillus subtilis, Leuconostoc mesenteroides, Pediococcus halophilus, Sarcina litoralis* and other salt resistant aerobic and anaerobic bacteria (Lee, 1993). Those bacteria in jeogal could function as probiotics in animal intestine by suppressing harmful bacteria including pathogens, and would substitute antibiotics that are used as additives in animal feeds (Fernandez et al., 2003; Lee et al., 2001; Osullivan, 2001). The research and development of food utilizing the functionality and effectiveness of probiotic has been recognized as an important field. Consequently, numerous studies on probiotic organism for production of functional foods have been performed (Tannock, 1997; Todorov et al., 2011; Leong et al., 2013). LAB are the most generally used probiotics (Naidu et al., 1999; Osullivan, 2001). The other benefit of LAB is to preserve the nutritive qualities of raw material of food and inhibit the spoilage and growth of pathogenic bacteria (Matilla-Sandholm et al., 1999) in processed foods.

Some LAB of *Lactobacillus* sp. and *Lactococcus* sp. were isolated from jeotgal and characterized for application as probiotic and bacteriocin producer (Jeun

et al., 2004; Kim et al., 1999; Kim et al., 2005; Lee et al., 2003). However, LAB isolated from jeotgal have not been studied enough and more study on LAB of jeotgal is required. This study was performed to isolate LAB producing antimicrobial substance from jeotgal, characterize the isolated LAB as a probiotic.

Materials and Methods

Screening of lactic acid bacteria producing antimicrobial substance

Jeotgal samples were collected at local markets in Korea. The jeotgals sampled were squid jeotgal, hairtail fish organ jeotgal, small octopus jeotgal, anchovy jeotgal and shrimp jeotgal. For isolation of LAB, the jeotgal was homogenized, serially diluted ten-fold with saline solution, plated on MRS agar(Rogosa. co.) and M 17 agar(Oxoid CM0325), and incubated at 37℃ for 2 to 3 d. Colonies formed on MRS agar and M 17 agar were picked randomly, and propagated on the same media until the pure cultures were obtained. All samples were kept at -80℃ in MRS broth containing 20 % (v/v) glycerol (Mathara et al., 2004) until analysis. Gram positive, catalase negative and facultative anaerobic bacteria were presumptively identified as LAB according to Gerhartdt et al.(1981). The isolated LAB were tested for the production of antimicrobial substance as antimicrobial activity in cell free culture supernatant. TTC test and paper disc diffusion method (Tadesse et al., 2005; Hernnadez et al., 2005) were employed to test the antimicrobial activity in cell free culture supernatant. Cell free culture supernatant was prepared by centrifugation (7500×g, 5 min, 4℃) of culture grown in MRS broth at 37℃ for 48 h, adjustment to pH 7.0 with 1 N NaOH, and filtration through 0.45μm pore Millipore membrane filter (Sharpe et al., 1979). *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* were used as the indicator strains in TTC test. Those indicator strains were cultured in trypticase soy broth, added to the cell free culture supernatant of the isolated LAB containing tetrazolium red (0.2 %), and incubated at 37℃. Antimicrobial activity against those indicator strains was identified by consistancy of tetrazolium red color after incubation for 16 h. *Listeria monocytogenes* was used as the indicator strain in paper disc diffusion method. Tryptic soy soft agar (0.7 %, w/v) inoculated with 1 % (v/v) of *Listeria monocytogenes* overnight culture, was overlaid on tryptic soy agar (1.5 %, w/v) in plate. And paper disc impregnated with the respective cell free culture supernatant and air dried was laid on the tryptic soy soft agar. The plate was incubated for 24 h at 37℃, and antimicrobial activity was identified as the formation of inhibitory zone around paper disc.

Identification of selected lactic acid bacteria

Identification of the selected isolate was performed by 16S rDNA sequence. The 16S rDNA was sequenced by the method described by Rainey *et al*. (1996) using the Big Dye terminator cycle sequencing kit (Applied Biosystems model 3730XL, Applied BioSystem). The 16S rDNA sequence of the strain was aligned to the 16S rDNA gene sequence of other LAB and related taxa in GenBank, and phylogenetic tree for the dataset was built by the neighbor-joining method (Saitou and Nei, 1987). To get some information on practical usefulness, biochemical properties were tested on mainly enzyme activities and carbohydrates utilization. Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) was used for the criteria of testing biochemical properties. The ability to ferment carbohydrate substrates was studied using the API 50 CH and API 50 CHL medium (Biomerieux, Lyan, France) system.

Viability of selected strain at gastric conditions, and resistance to heat

To determine the resistance to acidic condition of stomach, viable cells were enumerated during incubation for 120 min at 37℃ in MRS broth adjusted to pH 2.0 using 0.1 N HCl. The number of viable cells was determined by plating sample on MRS solid medium and incubating at 37℃ for 48 h. The resistance of selected strain to bile salts secreted into duodenum was tested by the growth of the selected strain in the presence of oxgall (Difco, Detroit, USA) added to the concentration of 3%(w/v) by the method described by Walker and Gilliland (1993). The growth was monitored during incubation at 37℃ statically up to 7h by optical density at 600nm(A_{600}). Heat resistance of the selected isolate was tested at 50℃ and 60℃. The isolate was cultured in MRS broth for 24h at 37℃ statically, and then was heated at 50 and 60℃ for 30 min. After heat treatment, viable cells were counted by plating the heated culture on MRS solid medium.

Hydrophobicity of cell surface

Determination of cell surface hydrophobicity was performed by testing the adherence of solvents of hydrocarbon to cell surface (Rosenberg et al., 1980). Cells of the isolated strain cultured at 37℃ in LAPTg broth were harvested by centrifugation (10000×g, 10 min at 4℃) at the early logarithmic growth phase(15 h of incubation time), washed twice and resuspended in physiological saline solution to the A_{600} of 0.6. To the test tube containing 3 ml of washed cells, one ml of hydrocarbon(hexadecane, toluene or xylene) was added, blended for 90 s on a vortex mixer, and left to stand for 15 min until phases of hydrocarbon and aqueous were separated. A_{600} of the aqueous phase was measured, and hydrophobicity was calculated as % microbial adhesion to solvent by the percentage decrease in the A_{600} of original bacterial suspension due to partitioning of cells into the hydrocarbon layer. *Mycobacterium* sp. was used as positive control and *Lactobacillus acidophilus* CRL 730 as negative control (Morata de et al., 1999).

% microbial adhesion to solvent =

[(A_{600} of original suspension–A_{600} of aqueous phase)/ A_{600} of original suspension]×100

Verification of antimicrobial substance by SDS-PAGE and proteolytic hydrolysis

The antimicrobial substance in cell free culture supernatant of selected isolate was verified by tricin-SDS-PAGE as described by Schagger and Von Jagow (1987), using 4% acrylamide of concentration gel and 15% acrylamide of separation gel. Two identical samples of cell free culture supernatant were electrophoresed in a same gel, and the gel was cut in two ones of each sample. One was stained with Coomassie brilliant blue R-250 for verification of protein. To identify antimicrobial substance in gel, the other gel was immersed for 2 h in the mixed aqueous solution of isopropanol(20%) and acetic acid(10%) to fix proteins in gel, rinsed with distilled water three times (initial rinse for 1 h followed by two washes for 5 min each), overlaid with 20 ml of soft Muller Hinton agar(0.7%) seeded with 5logCFU/ml of *Listeria monocytogenes*, and incubated at 37℃ for 16 h (Bhunia et al., 1987). Antimicrobial substance was identified by antimicrobial activity demonstrated by the presence of an inhibitory zone. Molecular Mass Markers for Peptides (Sigma) were used for Mw standards. Cell free culture supernatant of selected isolate was treated by proteases and tested for residual antimicrobial activity to examine if the antimicrobial substance is a proteineous one. Proteinase K or trypsin (Sigma, St. Louis, U.S.A.) dissolved in tris-HCl buffer (0.05 M, pH 8.0) was added to cell free culture supernatant adjusted to pH 8.0 by 1 N NaOH, and treated for 2 h at 37℃. The cell free culture supernatant treated by proteolytic enzymes was heated at 80℃ for 10 min to inactivate the enzyme, neutralized to pH 7.0 by 1 N HCl, and residual antimicrobial activity against *Listeria monocytogenes* was measured by paper disc diffusion method. Proteinase K of trypsin was added to the final concentration of 1 mg/ml in reaction solution.

Influence of heat, pH and amylolytic hydrolysis on antimicrobial substance

Effect of heat, pH and amylolytic hydrolysis on antimicrobial substance was chased by the change of antimicrobial activity against *Listeria monocytogenes* measured by paper disc diffusion method. Cell free culture supernatant of selected isolate was heated at various temperatures of 30 to 100℃ for 30 min, or autoclaved for 15 min at 121℃, and the effect of heat at each temperature was measured by the residual antimicrobial activity. Influence of pH on antimicrobial substance was assayed at pH 2 to 10. Cell free culture supernatant adjusted to pH 2 to 10 using 1 N HCl or 1 N NaOH (Hernnadez et al., 2005) was kept at 37℃ for 1 h, then pH of the cell-free culture supernatant was neutralized to pH 7.0, and the residual antimicrobial activity was measured. To determine the influence of amylolytic hydrolysis on antimicrobial substance, α-amylase (Sigma, St. Louis, U.S.A.) dissolved in Na-phosphate buffer (0.1 M, pH 7.0) was added to cell free culture supernatant adjusted to pH 7.0, incubate at 37℃ for 2 h, heated at 80℃ for 10 min to inactivate the enzyme, and the residual antimicrobial activity was determined. The final concentration of α-amylase in reaction solution was 1 mg/ml.

Results and Discussion

Screening of LAB producing antimicrobial substance and identification of selected strain

A total of 590 bacterial isolates were taken from jeotgal samples. Among them, 156 isolates were

screened as LAB by the properties of positive in Gram stain, non-spore formation, and negative in catalase activity. The 156 isolates were tested for the antimicrobial activity by TTC and selected finally by the inhibition activity confirmed by paper disc diffusion assay. The isolate KNOUC4036 showed the highest antimicrobial activity, and was selected. A phylogenetic tree derived from the 16S rDNA sequence (GenBank KF734671) showed the phylogenetic position of this isolate to closely related species as *Weissella halotolerans* DSM1529 with 98% similarity(Fig. 1). Therefore, the selected strain was identified and named as *Weissella halotolerans* KNOUC4036. The isolate KNOUC4036 produced some enzymes and utilized many carbohydrates (Table 1). The phylogeny of bacteria groups presently in genus *Weissella* was clarified in 1990 (Martinez-Murcia and Collin, 1990), and the taxonomy of *Weissella* species was further assessed in 1993 (Collins et al., 1993). They have been isolated from various sources, such as fresh vegetables (Wang and Nishinno, 2008), fermented silage (Ennaharr et al., 2003), meat or meat products (Santos et al., 2005), sugar cane, carrot juice, row milk and sewage (Hammes and Vogel, 1995), kimchi (Kim and Chun, 2005), fermented mescal of *Agave salmiana* (Escalante-Minakata et al., 2008), honey in Malaysian (Tajabadi et al., 2012) and Taiwan food (Leong et al., 2013), and some of them were reported to play significantly important roles in fermentation (Björkroth et al., 2002). However, *Weissella* associated with fermented fish has not been reported. There have been only a few preliminary studies on antimicrobial substances from *Weissella* genus (Papathanasopoulos et al., 1997; Srionnual et al., 2007; Papagianni and Papamichael, 2011), and bacteriocin from *Weissella halotolerans* has not been reported yet.

Table 1 Physiological and biochemical properties of strain KNOUC4036

Tests		**Tests**	
Gram's stain	+	Galactose	+
Catalase test	-	D-Glucose	+
Oxidase test	-	D-Fructose	+
Spore formation	-	D-Mannose	-
Shape	Rod	L-Sorbose	-
Enzyme test		Rhamnose	-
Alkaline phosphate	-	Dulcitol	-
Esterase	+	Inositol	-
Esterase lipase	+	Mannitol	-
Lipase	-	Sorbitol	-
Leucine arylamidase	-	A-Methyl-D-mannosise	+
Valine arylamidase	-	a-Methyl-D-glucosid	-
Cystine arylamidase	-	N-Acetyl glucosamine	-
Trypsin	-	Amygdalin	-
α-Chymotrypsin	-	Arbutine	+
Acid phosphatase	+	Esculin	-
Naphtol-AS-BI-phosphohysrolase	-	Salicine	-
α-galactosidase	-	Cellobiose	+
β-galactosidase	-	Maltose	-
β-glucoronidase	-	Lactose	+
α-fucosidase	-	Melibiose	+
α-mannosdiase	-	Saccharose	+
N-acetyl-β-glucosamidase	-	Trehalose	-
Carbohydrate utilization		Inuline	-
D-Arabito	-	melezitose	+
Glycerol	-	D-Raffinose	-
2keto-gluconate	-	Amidon	-
5keto-gluconate	-	Glycogen	-

Erythriol	-	Xylitol	-
D-Arabinose	-	b-Gentiobiodr	+
L-Arabinose	+	D-Turanose	-
Ribose	+	D-Lysoxe	-
D-Xylose	-	D-Tagatose	-
L-Xylose	-	D-Fucose	-
Adonitol	-	L-Fucose	-
B-Methyl-xyloside	-		
L-Arabitol	-		
Gluconate	-		

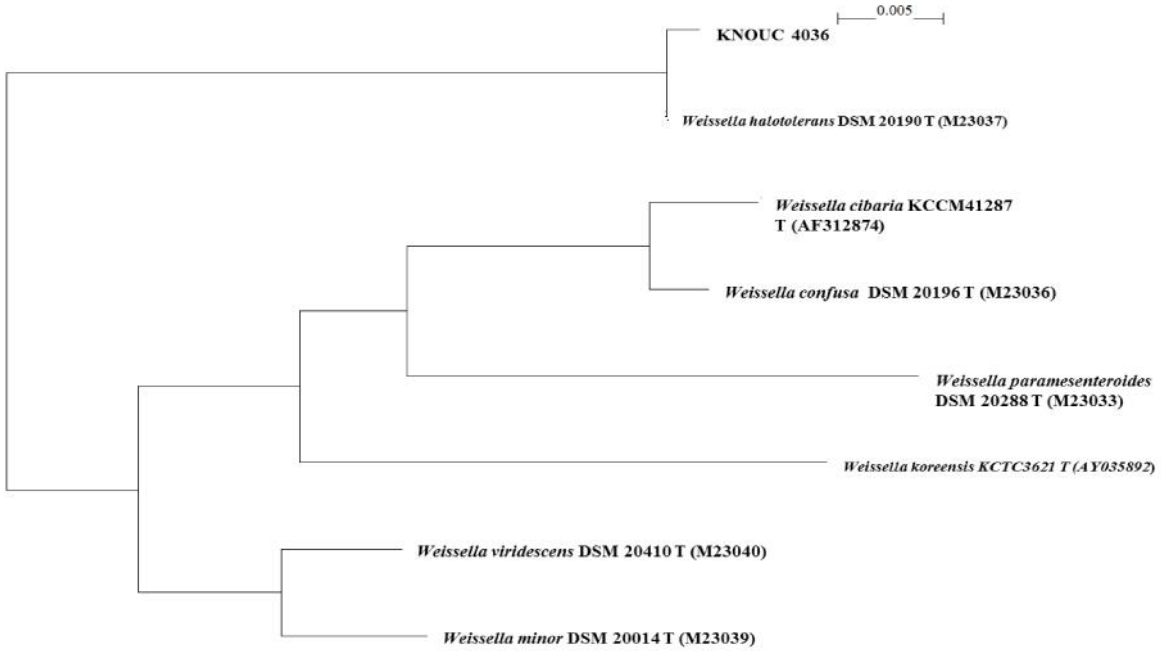

Fig. 1 Phylogenetic tree based on 16S rDNA sequences showing the positions of strain KNOUC4036, the type strains of *Weissella* species and the representative of some other related taxa.
*Scale bar represents 0.005 substitution per nucleotide position

Resistance of selected strain KNOUC4036 to gastric conditions and heat

The acidic condition of pH 2 in stomach (Morelli, 2000; Yildirim et al., 2002) and bile salts of 0.3% in gastrointestinal track (Papamanoli et al., 2003) are the harsh circumstances that the microorganisms orally taken as probiotic have to survive while passing through for about 4 h (Prasad et al., 1998) to arrive and colonize at intestinal surface. During acid treatment of the isolate KNOUC4036 performed at pH 2.0 for 2 h at 37℃, among initial 8.41log CFU/ml, 6.07log CFU/ml and 5.73log CFU/ml survived in 60min and 120min respectively (Table 2). Tolerance of the isolate KNOUC4036 to bile salts was determined by the growth of cells in MRS broth containing oxgall at 3%, the physiological concentration of bile salts in small intestine (Gunn, 2000). As shown in Table 3, the oxgall added in MRS broth did not affect the growth of the isolate KNOUC4036. The resistance at pH 2 (Table 2) and no harm by oxgall of 0.3 % (Table 3) suggest that Weissella halotolerans KNOUC4036 will pass well through the harsh gastric environment to intestinal track. Probiotic stable at high temperature is preferable in preparation of commercial product and delivery to customer. When 9.43 log CFU/ml of the isolate KNOUC4036 was heated at 50 or 60℃ for 30 min, 8.45logCFU/ml and 8.27logCFU/ml survived respectively as in Fig. 2, showing fair stability.

Table 2 Acid tolerance of *Weissella halotolerans* KNOUC4036

Treatment time at pH2.0	Viable cell counts (log CFU/ml) after treatment at pH2.0
0min.	8.41±0.11
15min.	7.82±0.23
30min.	7.10±0.30
45min.	6.53±0.15
60min.	6.07±0.06
120min.	5.73±0.10

*Cells were treated at 37℃ in MRS broth adjusted to pH 2.0

.

Table 3 Bile acid tolerance of *Weissella halotolerans* KNOUC4036

Incubation time	Growth of cells Control	(log CFU/ml)*3% oxgall
0h	0.216±0.005	0.258±0.015
1h	0.441±0.009	0.435±0.016
2h	0.526±0.015	0.544±0.018
3h	0.679±0.004	0.715±0.006
4h	0.858±0.015	0.902±0.008
5h	1.005±0.005	1.039±0.011
6h	1.080±0.013	1.137±0.006

*Cells were grown at 37℃ in MRS broth(control) or MRS broth added by oxgall at the concentration of 3%(3% oxgall).

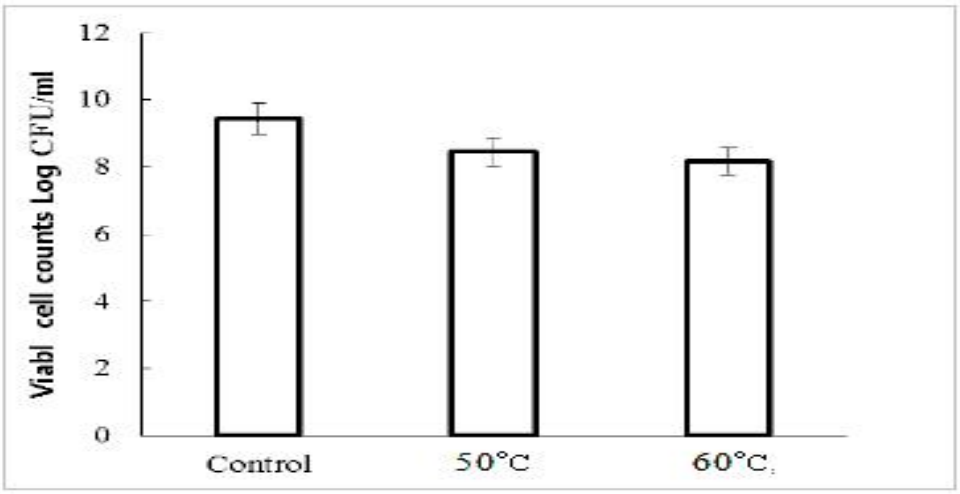

Fig. 2 Effect of heat treatment on *Weissella halotolerans* KNOUC4036

*Cells cultured in MRS broth were heated for 30 min. at 50℃ or 60℃, and viable cells were counted.

Hydrophobicity of selected strain KNOUC4036

Hydrophobicity of cell surface of bacteria is one of factors that determine the adhesion of bacterial cells to host intestinal surface (Ram and Chander, 2003), and cell of higher hydrophobicity is likely to have more opportunity to inhabit in the human gastrointestinal tract (Naidu et al., 1999).

In vitro adhesion of microorganism to organic solvents was reported to be qualitatively valid to estimate the ability of microorganisms to adhere to epithelial cells(Kiely and Olson, 2000). The hydrophobicity, expressed as % microbial adhesion to solvent, of isolate KNOUC4036 to hexadecane, toluene and xylene were 79.96%, 69.51% and 60.37%, respectively as shown in Fig 3, indicating high potential to adhere to gut epithelial cells of human intestine and colonize there (Ram and Chander, 2003). Hydrophobicity of *Weissella halotolerans* KNOUC4036 was higher than those of *Lactobacillus delbrueckii* (43.7%), *Pediococcus acidilactici* (51.3%) and *Lactobacillus rhamnosus* GG (53.3%), and was similar with those of *Lactobacillus curvatus* (61.9–64.6%) and *Lactobacillus fermentum* (78.9%) (Todorov et al., 2011).

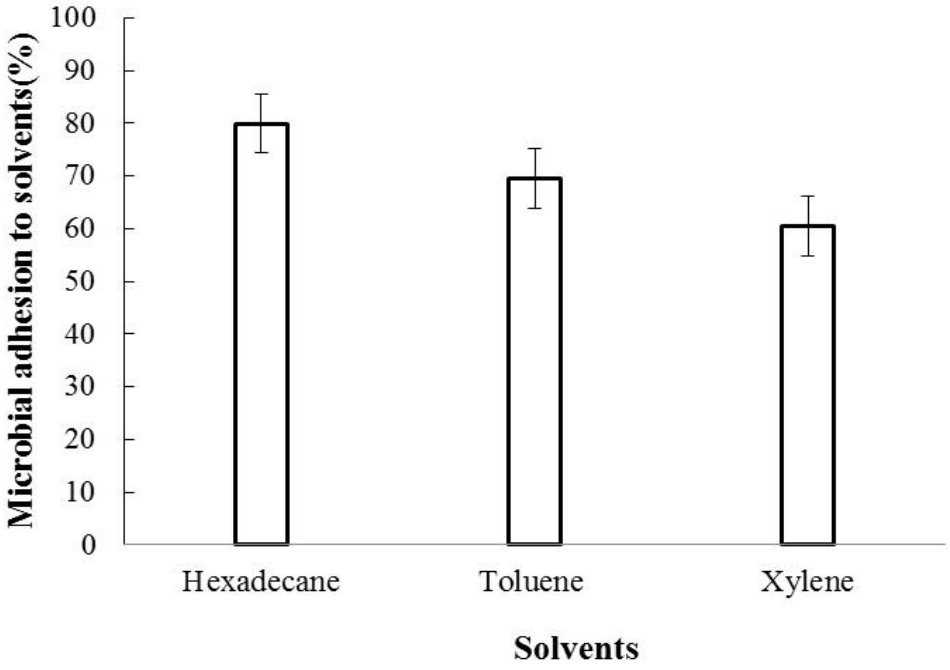

Fig. 3 Hydrophobicity of *Weissella halotolerans* KNOUC4036 against various solvents

* % Microbial adhesion to solvent (hydrophobicity)
= [(A_{600} of original suspension – A_{600} of aqueous phase)/ A_{600} of original suspension] ×100

Antimicrobial substance in cell free culture supernatant of selected strain KNOUC4036 confirmed by SDS-PAGE

Cell free culture supernatant of isolate KNOUC4036 cultivated in MRS broth was examined on SDS-PAGE to find the existence of antimicrobial substance by comparing protein band stained by Coomassie brilliant blue and microbial inhibitory zone formed against *Listeria monocytogenes* by soft agar overlay method. As in Fig. 4, the position of main protein band in the gel stained with Coomassie brilliant blue coincided with the inhibitory zone formed by the soft agar overlay method. The antimicrobial substance was shown at the region smaller than 7 kDa. Proteolytic hydrolysis of cell free culture supernatant of strain KNOUC4036 by protease K and trypsin extinguished antimicrobial activity assayed by disc diffusion method performed against *Listeria monocytogenes* (Table 4), indicating that the antimicrobial substance is a proteinous one. Complete inactivation of the antimicrobial substance's antimicrobial activity by proteolytic hydrolysis implys advantages such as no toxic, no immune problem, and no harm to microflora in large intestine because it is degraded by proteinase in gastrointestine. The molecular mass of strain KNOUC4036 antimicrobial substance was smaller than 7 kDa, similar with those of bacteriocins produced by *Weisella cibaria* 110 (Srionnual et al., 2007) and *Weisslla hellenica* 4-7 (Leong et al., 2013).

Table 4 Effect of hydrolytic enzymes, pH and heat treatments on antimicrobial activity of antimicrobial substance in cell free culture supernatant of *Weissella halotolerans* KNOUC4036

Treatment		**Antimicrobial activity***
Enzyme	α-amylase	-
	protease K	-
	trypsin	-
pH	2	12.13±0.15
(at 37℃, 1hr)	4	13.37±0.25
	5	13.17±0.31
	6	13.20±0.20
	7	13.10±0.10
	8	13.30±0.10
	10	-
Temp.	30℃ 30min	13.30±0.26
	40℃ 30min	13.06±0.30
	50℃ 30min	13.16±0.31
	60℃ 30min	13.20±0.20
	70℃ 30min	13.20±0.20
	80℃ 30min	13.30±0.10
	90℃ 30min	13.20±0.20
	100℃ 30min	13.17±0.21
	121℃ 15min	13.30±0.26

* Antimicrobial activity was determined as the inhibition zone(mm) against indicator strain, *Listeria monocytogenes* by disc diffusion method.

(-) : no inhibition zone against indicator strain , *Listeria monocytogenes* by disc diffusion method

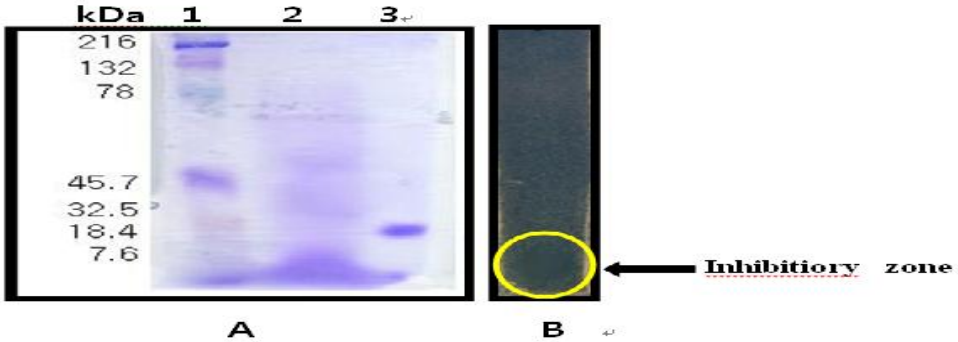

Fig. 4. SDS-PAGE and antimicrobial activity of the cell-free supernatant of *Weissella halotolerans* KNOUC4036

A. Gel stained with Coomasssie Brilliant Blue G250. 1. molecular weight standards (broad range, BIO-RAD #161-031) 2. cell free culture supernatants of *Weissella halotolerans* KNOUC4036 3. molecular weight standards (polypeptide SDS-PAGE standards, BIO-RAD #161-0326) B. Gel overlaid with MH agar containing indicator organism, *Listeria monocytogenes*. Inhibition zone formed by the antimicrobial substance in cell free culture supernatants of *Weissella halotolerans* KNOUC4036 is indicated by an arrow.

Stability of antimicrobial substance produced by selected strain KNOUC4036 to heat, pHs and amylolytic hydrolysis

Antimicrobial substance has to be stable to heat and at wide range of pH for practical usefulness. Antimicrobial substance of strain KNOUC4036 retained full activity at the treatment for 30 min at 100℃ and even at autoclaving at 121℃ (Table 4, Fig 5) similar with weissellicin L produced by *Weissella hellenica* 4-7 (Leong et al., 2013), and weissellin A produced by *Weissella paramesenteroides* DX (Papagianni and Papamichael, 2011). Antimicrobial substance of strain KNOUC4036 was stable at pH 2 to 8 for 1 h at 37℃, but lost its activity by the exposure to pH 10 (Table 4), showing that the extremely alkaline condition is not safe for it. Weissellicin Y produced by *Weissella hellenica* QU13 was inactivated at alkaline pH (Masuda *et al*. 2012). The antimicrobial substance of isolate KNOUC4036 lost all of its antimicrobial activity against *Listeria monocytogenes* by the hydrolysis with α-amylase (Table 4). This result implies that the essential moiety for antimicrobial activity of the antimicrobial substance was destroyed by α-amylase.

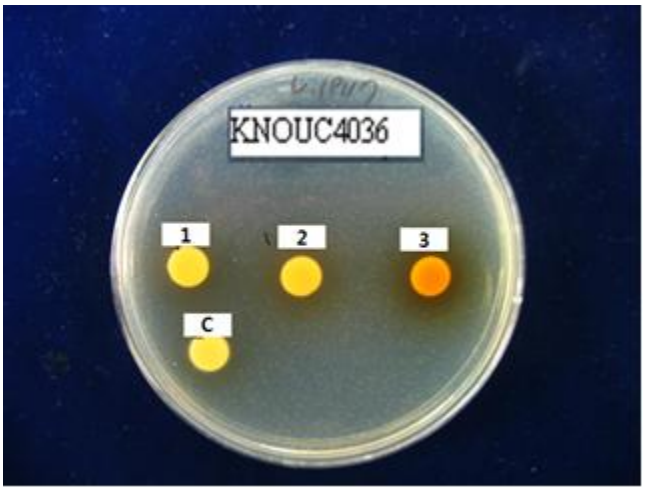

Fig. 5 Antimicrobial activity of cell free culture supernatants of KNOUC4036 after heat treatment

* Antimicrobial activity was measured against Listeria monocytogenes ATCC 19117 by agar well diffusion assay.
* Cell free culture supernatants was heated for 30 min. at 90℃(1) and 100℃(2), or autoclaved at 121℃ for 15min(3). Control (C) is the cell free culture supernatant unheated.

Conclusion

The *Weissella halotolerans* KNOUC4036 was fairly stable at the pH of stomach and to oxgall, showed high hydrophobicity, and produced a proteinous antimicrobial substance that is stable at wide range of pH and temperature. Considering those properties above, in conclusion this strain is supposed to have a high potential as a probiotic.

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