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Isolation of *Lactobacillus curvatus* KNOUC4148 from Kimchi and Its Properties as a Probiotic

E. S. Nam[1] and J. K. Ahn[2]*

[1]Sogang-Binggrae Food Advanced Analysis Center, Sogang University, Seoul 121-742, Republic of Korea
[2]Department of Agricultural Sciences, Korea National Open University, Seoul 110-791, Republic of Korea
**Corresponding author*

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A B S T R A C T

This study was to screen lactic acid bacteria useful for probiotics from a Korean fermented food, kimchi. A strain KNOUC4148 was selected and identified phenotypically and genotypically as *Lactobacillus*(*Lb.*) *curvatus*, and named as *Lb. curvatus* KNOUC4148. The strain showed acid tolerance retaining viability of 4.82 $\log_{10}$ CFU/ml among initial 8.15 $\log_{10}$ CFU/ml after treatment at pH 2 for 120min. Oxgall added in MRS broth at the concentration of 3% did not inhibit the growth of *Lb. curvatus* KNOUC4148. Cell surface hydrophobicity of *Lb. curvatus* KNOUC4148 was 71.89 - 86.25% to organic solvents of hexadecane, toluene and xylene. The antimicrobial activity of antimicrobial substance produced by the isolate kept unchanged through acidic or alkaline treatment of pH 2 to pH10, and heat treatment at 30°C to 100°C for 30min and at 121°C for 15min. The antimicrobial substance of KNOUC4148 lost completely its antimicrobial activity by proteolytic and amylolytic hydrolysis. SDS-PAGE analysis revealed an apparent antimicrobial substance.

Introduction

Kimchi, a Korean traditional fermented vegetable food made of vegetables and seasonings including napa cabbage, radish, hot red pepper, green onion, garlic and ginger, is recognized as functional food due to its beneficial effects on human health derived from components in raw materials, fermentation products and lactic acid bacteria (LAB) (Kim *et al.*, 2011).

Particularly LAB are attracting interest for immune stimulation, pathogen exclusion, production of bioactive substances and general intestinal health (Naidu *et al.*, 1999). In addition, LAB impart a characteristic flavor to fermented foods (Lui *et al.*, 2008) and increase their shelf life by protecting foods from many kind of bacteria including putrefactive bacteria by producing inhibitory

agents such as organic acids, hydrogen peroxide, ethanol, reuterin and bacteriocin(Ross *et al.,* 2002). Especially LAB bacteriocin has possibilities to prevent the development of specific unwanted spoilage and pathogenic bacteria in both of fermented and non-fermented foods because of their broad- or narrow-host-range specificity(Ross *et al.,* 2002). LAB bacteriocins were produced by *Lb*., *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* spp. (Naidu *et al.,* 1999: Todorov *et al.,* 2011). Regarding the application of bacteriocin-producing starter strains in food fermentation, the major problem is related to the in situ antimicrobial efficacy which can be negatively influenced by various factors, such as binding of the bacteriocins to food components (fat or protein particles) and food additives(egg, triglyceride oils), inactivation by proteases or other inhibitors, changes in solubility and charge, and changes by the cell envelope of target bacteria(Aasen *et al.,* 2003: Leroy and De Vuyst, 1999).

Kimchi is growing in popularity throughout the world partly because of its LAB inhibiting intestinal pathogens. LAB producing bacteriocin in kimchi are targeted to be investigated(Han *et al.,* 2013), and it is necessary to find more LAB producing antimicrobial substance to explain the function of kimchi and to get LAB useful for probiotics and food industry. Accordingly this study was to screen and identify LAB producing bacteriocin like antimicrobial substance from kimchi and to characterize the properties of the isolate as a probiotic.

Materials and Methods

Isolation of lactic acid bacteria producing antimicrobial substance

Kimchi samples, baechu kimchi and kkakdugi available at local markets in Korea, were homogenized, serially diluted ten-fold with saline solution, plated on MRS agar(Difco, Detroit, USA) containing Na-azide(0.02%), and incubated statically at 37℃ for 2-3 days. Colonies formed on MRS agar were randomly selected and propagated until the pure cultures were obtained. The isolates of Gram reaction positive, catalase negative, and non-spore former were recognized as presumptive LAB, and chosen for further study. The antimicrobial activities in cell free culture supernatants of LAB isolated were tested by modified triphenyltetrazolium chloride(TTC) test (Summanen *et al.,* 1992) and paper disc diffusion method (Hernnadez *et al.,* 2005). Cell free culture supernatants were prepared by centrifugation(7,500×g, 5min, 4℃) of culture grown in MRS broth statically at 37℃ for 48 h, and filtered through Millipore membrane filters(0.45μm pore) after the pH of the supernatants was adjusted to pH 7.0 with 1 N NaOH. TTC test was performed against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*.

Those indicator strains cultured in trypticase soy broth was added to the cell free culture supernatants containing tetrazolium red(0.2%), incubated for 16 h at 37℃, and antimicrobial activity against those indicator strains was identified by invariance of tetrazolium red color. For the test of antimicrobial activity on paper disc diffusion method, *Listeria monocytogenes* was used as the indicator strain. 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). Paper discs impregnated with the respective cell free culture supernatants and air dried were laid on the tryptic soy soft agar in a plate prepared above. The plate was incubated for 24 h at 37℃, and examined for the formation of inhibition zone.

Identification of Isolated Strain

Identification of the selected isolate was performed by morphological and biochemical properties, and 16S rDNA sequence. Morphological and biochemical properties were tested according to the criteria of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Carbohydrate fermentation was studied using an API 50 CHI kit (Biomerieux, Lyan, France). The sequencing of 16S rDNA was carried out by the method of 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 LAB and other related taxa in Genbank to compare the levels of similarity, and phylogenetic tree for the dataset was constructed by the neighbor-joining method (Saitou and Nei, 1987).

Resistance of isolated strain to heat, acid and bile salt

The isolated strain cultured in MRS broth for 24 h at 37℃ statically, was heated at 50℃ and 60℃ for 30 min. The number of viable cells were determined by plating the heat treated culture on MRS agar and incubating at 37℃ for 48 h. To determine the resistance to acidic conditions, viable cells were enumerated during incubation for 120 min at 37℃ in MRS broth adjusted to pH 2.0 using 0.1N HCl. Viable cell counts were performed by plating on MRS agar. To study the resistance to bile salt, effect of oxgall on the growth of the isolate was tested by the method described by Walker and Gilliland(Walker and Gilliland, 1993). Culture of the isolate was inoculated in MRS broth containing oxgall(Difco, Detroit, USA) added to the concentration of 0.3% (w/v), and cultured at 37℃. The growth at 37℃ was monitored by OD_{600nm} readings (D_{600}) up to 7 h using spectrophotometer.

Cell surface Hydrophobicity of Isolated Strain

Cells of the isolate grown at 37℃ in LAPTg broth were harvested by centrifugation(10,000×g, 10 min, 4℃) at the early logarithmic growth phase(12-18 h of incubation), washed twice with physiological saline solution, resuspended in the same solution to the D_{600} of 0.5-0.7, and used for assay. Cell surface hydrophobicity was determined by the adherence to solvents of hydrocarbon (hexadecane, toluene or xylene) by the method of Kiely and Olson(2000). One ml of hydrocarbon was added to the 3 ml of washed cells in test tube.

The mixture was blended on a vortex mixer for 90 sec, then the tube was left statically for 15 min until phases of aqueous and hydrocarbon were separated, and D_{600} of the aqueous phase was measured. Hydrophobicity was calculated as the percentage decrease in the D_{600} of original bacterial suspension due to partitioning of cells into the hydrocarbon layer. *Mycobacterium* sp. was used as positive control and *Lb. acidophilus* CRL 730 as negative control. Hydrophobicity was indicated as % microbial adhesion to organic solvents as follows.

% microbial adhesion to solvent = [(D_{600} of original suspension – D_{600} of aqueous phase)/ D_{600} of original suspension] × 100

Effect of heat, pH and enzymatic hydrolysis on antimicrobial activity of antimicrobial substance.

Cell free culture supernatant was used for assay, and antimicrobial activity was

determined by disc diffusion method. To test the effect of heat, the cell free culture supernatant of the isolate was exposed to various temperatures at 30, 40, 50, 60, 70, 80, 90 and 100℃ for 30 min, or autoclaved for 15 min at 121℃, and the residual antimicrobial activity was tested. Resistance to various pHs was assayed at pH 2 to pH 10. The cell free culture supernatant was adjusted to pH 2 to pH 10 using 1N HCl or 1N NaOH(Hernnadez *et al.,* 2005), and was incubated for 1 h at 37℃ at each pH. After the incubation, pH of the cell free culture supernatant was neutralized to pH 7.0 with 1N HCl or 1N NaOH and the residual antimicrobial activity was determined. To determine the influence of proteolytic and amylolytic enzymes on antimicrobial activity, cell free culture supernatant of the isolate was treated with proteinase K, trypsin or α-amylase(Sigma, St Louis, MO, USA) at 37℃ for 2 h. Proteinase K and trypsin were dissolved in tris-HCl(0.05M, pH 8.0), α-amylase was dissolved in Na-phosphate buffer(0.1M, pH 7.0), and the enzyme solutions were filtered through Millipore membrane filter(0.45μm pore). All the enzymes were used at the final concentration of 1mg/ml. After hydrolysis, the mixture of enzyme and cell free culture supernatant of the isolate was heated at 80℃ for 10 min to inactivate the enzymes, and assayed for residual antimicrobial activity.

Confirmation of antimicrobial substance on SDS-PAGE gel

The antimicrobial substance in cell free culture supernatant was confirmed by tricin-SDS-PAGE(Schagger and Von Jagow, 1987), using concentration gel of 4% acrylamide and separation gel of 15% acrylamide. After electrophoresis of two identical samples, the gel was cut in two vertical ones of each sample. One was stained with Coomassie brilliant blue R-250, and the other one was assayed for antimicrobial activity against *Listeria monocytogenes* by the soft agar overlay method according to Bhunia et al.(1987) with modification. Briefly, the gel was fixed in the mixed aqueous solution of isopropanol(20%) and acetic acid(10%) for 2 h, rinsed with distilled water(initial rinse for 1hr. followed by two washes of 5min. each), and overlaid with 20 ml of soft Muller Hinton agar(0.7%) seeded with 10^5 CFU/ml of *Listeria monocytogenes*. After incubation at 37℃ for 16 h, the gel was examined for the presence of an inhibitory zone.

Results and Discussion

Screening and Identification of LAB Producing Antimicrobial Substance

Three hundred and fifty one isolates were taken from the kimchi samples. Among them, 196 isolates were screened as presumptive LAB by the properties of positive in Gram stain, non-spore formation, and negative in catalase activity. The 196 isolates were tested for antimicrobial activity by TTC test and paper disc diffusion assay. In the test of antimicrobial activity, isolates KNOUC4148 and KNOUC4149 showed high activity. Those two selected isolates were tested for biochemical properties. As in Table 1, isolates KNOUC4148 and KNOUC4149 were presumably identified as *Lb. curvatus* and *Leuconostoc mesenteroides* respectively(Holt *et al.,* 1994). The isolate KNOUC4149 produced β-glucuronidase catalyzing the hydrolysis of benzopyrene-3-glucuronide to a benzopyrene derivative that is a carcinogenic substance(Kim and Jin, 2001), and the isolate KNOUC4148 was finally chosen and tested its 16S rDNA sequence for further identification. In the phylogenetic tree generated by 16S rDNA

sequence (GenBank: KF411435), the isolate KNOUC4148 formed a distinct cluster with *Lb. curvatus* DSM20019 in 99.8% similarity(Figure 1). Therefore, the isolate was finally identified and named as *Lb. curvatus* KNOUC4148. Many strains of *Lb. curvatus* were isolated from sausages(Mataragas *et al.,* 2002: Sudirman et al, 1993: Tichaczek *et al.,* 1992: Verluyten *et al.,* 2004), retail meats(Garver and Muriana, 1994), dairy food(Casla *et al.,* 1996), fermented sausage(Mataragas *et al.,* 2002: Verluyten *et al.,* 2004) and kimchi (Ha *et al.,* 1994: Kim *et al.,* 1998).

Properties of Isolate KNOUC4148 for Probiotic

LAB stable at high temperature is advantageous for practical use, and microorganisms used as probiotics taken orally have to pass the acidic condition of pH 2 in stomach(Morelli, 2000), and bile salts of 0.1-0.3% in gastrointestinal track with residence time of about 4 h(Gunn, 2000) to arrive and colonize at intestinal surface. As in Table 2, among 8.68 log10CFU/ml of the strain KNOUC4148 heated at 50 and 60℃ for 30min, 8.41 log10CFU/ml and 7.30 log10CFU/ml survived respectively, showing fair stability. In the acid tolerant test, among initial 8.15 log10CFU/ml, 6.27 log10CFU/ml and 4.82 log10CFU/ml survived at pH 2.0 in 60 min and 120 min respectively(Table 2). Oxgall of 0.3% in MRS broth did not affect the growth of isolate KNOUC4148(Figure 2). These acid and oxgall tolerance may suggest that *Lb. curvatus* KNOUC4018 will pass through the harsh gastric environment to intestinal track. To colonize at intestine, microorganism has to attach to intestinal surface. Cell hydrophobicity is one of factors that contribute to adhesion of bacterial cells to host tissues, and cell of higher hydrophobicity is likely to have more opportunity to inhabit in the human gastrointestinal tract(Naidu *et al.,* 1999). In vitro determination of microbial adhesion to organic solvents of hexadecane, toluene and xylene droplets was carried out. This method was reported to be qualitatively valid to estimate the ability of microorganisms to adhere to epithelial cells(Kiely and Olson, 2000). Hydrophobicities of the isolate KNOUC4148 to hexadecane, toluene and xylene were 71.89%, 81.95% and 86.25% respectively as shown in Figure 3. Hydrophobicity of *Lb. curvatus* KNOUC4148 was higher than those of *Lb. fermentum*(78.9%), *Lb. delbrueckii*(43.7%), *Pediococcus acidilactici*(51.3%), and *Lb. rhamnosus* GG(53.3%)(Todorov *et al.,* 2011). Surface proteins and lipoteichoic acids were reported to confer hydrophobic properties to the cellular surface of several strains of *Lb*. sp.(Schar-Zammaretti and Ubbink, 2003), and hydrophobicity of cell surface could be enhanced by modifying the composition and pH of cultivation media(Ram and Chander, 2003).

Properties of Antimicrobial Substance Produced by Isolate KNOUC4148

Stability to heat and at wide range of pH is required for antimicrobial substance to be used in food industry. The antimicrobial substance produced by isolate KNOUC4148 in cell free culture supernatant showed excellent stability by maintaining full antimicrobial activity at the treatment for 30min at 30 to 100℃ and at autoclaving for 15 min at 121℃(Figure 4 and Table 3). The bacteriocins produced by *Lb. curvatus* DU0162 and DU0182 lost 50% of activity in 30 min at 100℃ and were completely inactivated in 10 min at 121℃(Ha *et al.,* 1994).

Table.1 Physiological and Biochemical Properties of Strain KNOUC4148 and KNOUC4149

Characteristics	KNOUC 4148	KNOUC 4149	Characteristics	KNOUC 4148	KNOUC 4149
Gram's stain	+, rod	+, cocci	5keto-gluconate	-	-
Catalase test	-	-	D-Glucose	+	+
Oxidase test	-	-	D-Fructose	+	+
Spore formation	-	-	D-Mannose	-	-
			L-Sorbose	-	-
Enzyme test			Rhamnose	-	-
Alkaline phosphatase	-	+	Dulcitol	-	-
Esterase	+	+	Inositol	-	-
Esterase lipase	+	-	Mannitol	-	-
Lipase	-	-	Sorbitol	-	-
Leucine arylamidase	-	-	Methyl-α-D-mannoside	-	-
Valine arylamidase	-	-	Methyl-α-D-glucoside	+	+
Cytine arylamidase	-	-	N-Acetylglucosamine	-	-
Trypsin	-	-	Amygdalin	-	-
α-chymotrypsin	-	-	Arbutin	+	-
Acid phosphatase	+	+	Esculin	+	-
Naphtol-AS-BI-phosphohydrolase	+	-	Salicin	+	-
α-galactosidase	-	+	Gluconate	-	-
β-galactosidase	-	+	Xylitol	-	-
β-glucoronidase	-	+	Lactose	-	+
α-glucosidase	+	+	Melibiose	-	+
β-glucosidase	-	+	Saccharose	-	+
N-acetyl-β-glucosamidase	-	-	Trehalose	-	-
α-mannosidase	-	-	Inuline	-	-
α-fucosidase	-	-	Melezitose	-	+
			Galactose	+	+
Carbohydrate utilization			Amidon	-	-
Glycerol	-	-	β-Gentiobiose	-	-
Erythriol	-	-	D-Raffinose	-	-
D-Arabinose	-	+	D-Arabitol	-	-
L-Arabinose	+	-	D-Turanose	-	-
Ribose	+	+	D-Lyxose	-	-
D-Xylose	-	-	D-Tagatose	-	-
L-Xylose	-	-	D-Fucose	-	-
Adonitol	-	-	L-Fucose	-	-
β -Methyl-xyloside	+	-	L-Arabitol	-	-
2keto-gluconate	-	-			

Presumtive identification
KNOUC4148 : *Lactobacillus curvatus*
KNOUC4149 : *Leuconostoc mesenteroides*

+, positive reaction; -, negative reaction

Table.2 Heat Stability and Acid Tolerance of *Lactobacillus curvatus* KNOUC4148

Treatment	Viable cells (log_{10}CFU/ml)
Heat stability	
Control	8.68±0.03
50℃	8.41±0.25
60℃	7.30±0.28
Acid tolerance at pH 2.0	
0 min.	8.15±0.12
15 min.	7.64±0.27
30 min.	7.40±0.34
45 min.	7.01±0.20
60 min.	6.27±0.62
120 min.	4.82±0.27

Table.3 Effect of Hydrolytic Enzymes, pH and Temperature on Antimicrobial Activity of the Antimicrobial Substance in cell Free Supernatant of *Lactobacillus Curvatus* KNOUC4148

Treatment		Antimicrobial activity
Control		+[1,2]
Enzyme	α-amylase	-
	protease K	-
	trypsin	-
pH	2	(+)
(at 37℃, 1hr)	4	+
	5	+
	6	+
	7	+
	8	(+)
	10	(+)
Temp.	30℃ 30min.	+
(at pH 7.0)	40℃ 30min.	+
	50℃ 30min.	+
	60℃ 30min.	+
	70℃ 30min.	+
	80℃ 30min.	+
	90℃ 30min.	+
	100℃ 30min.	+
	Autoclaving(121℃, 15min.)	+

[1] Antimicrobial activity was determined as the inhibition of the growth of indicator strain, *Listeria monocytogenesis* by disc diffusion method;

[2] - : no growth of indicator strain, + : growth of indicator strain, (+) : weak growth of indicator strain

Fig.1 Phylogenetic tree based on 16S rDNA sequences showing the positions of strain KNOUC4148, the type strains of Lactobacillus species and the representative of some other related taxa. Scale bar represents 0.02 substitution per nucleotide position

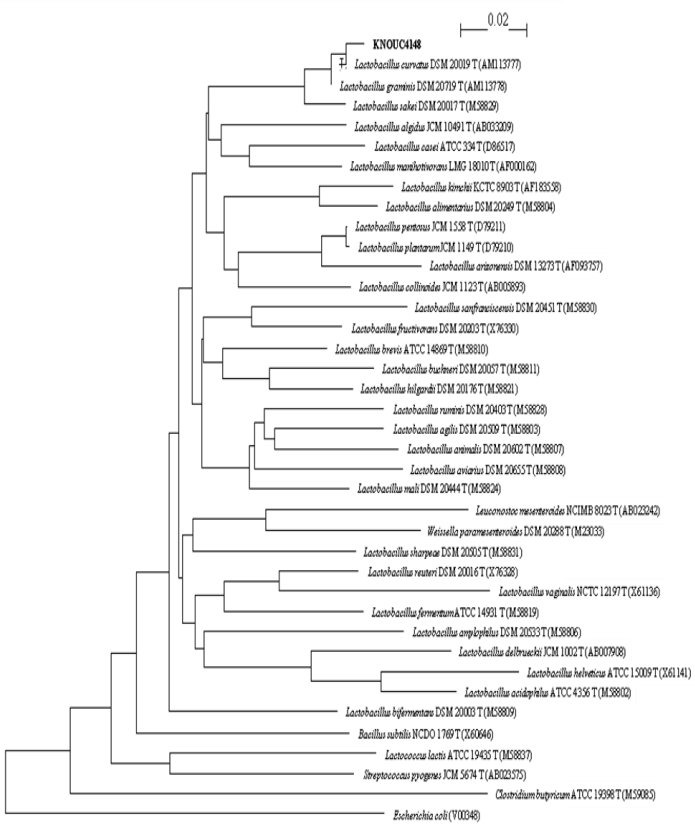


Fig.2 Effect of 0.3% oxgall in MRS Broth on Growth of *Lactobacillus curvatus* KNOUC4148 as Determined by OD at 600nm(D600)

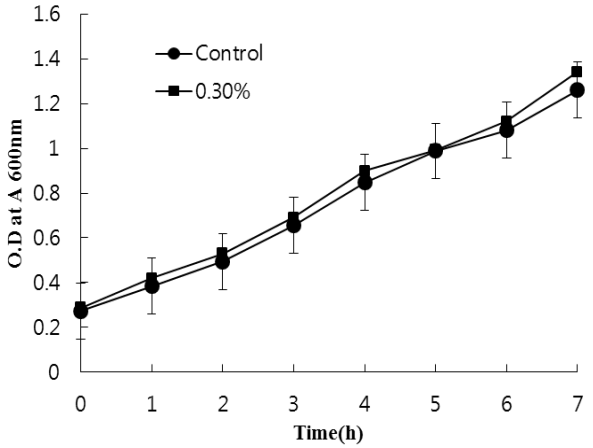
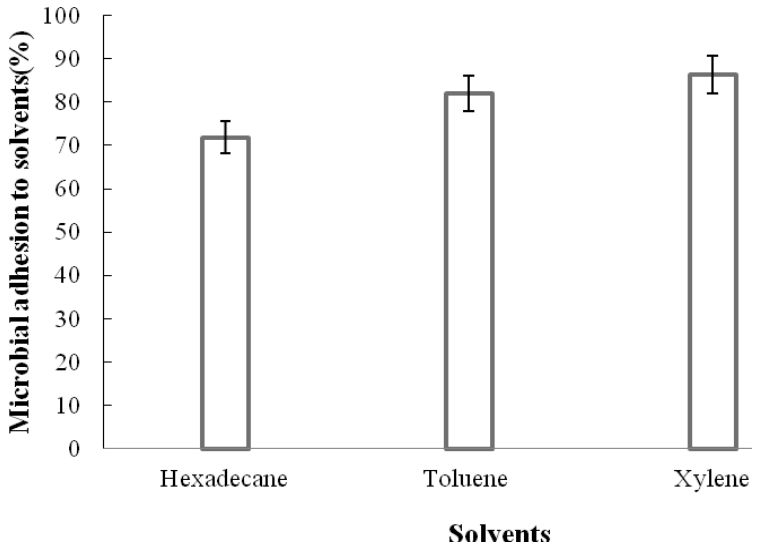
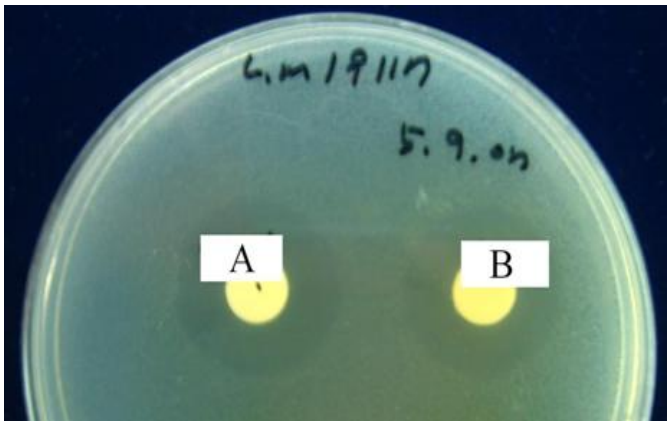


Fig.3 Hydrophobicity of *Lactobacillus curvatus* KNOUC4148 against various Solvents



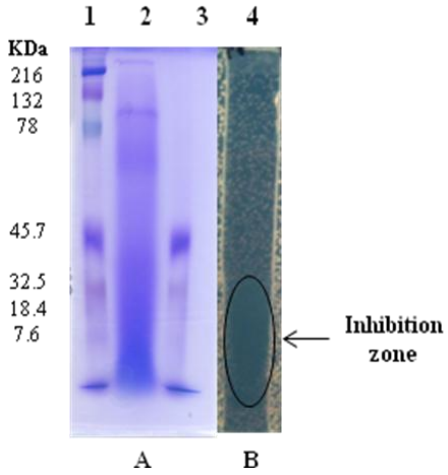
* % Microbial adhesion to solvent (hydrophobicity)
= [(A_{600} of original suspension – A_{600} of aqueous phase)/ A_{600} of original suspension] ×100

Fig.4 Antimicrobial Activity of Cell-free Supernatant of *Lactobacillus curvatus* KNOUC4148 against *Listeria monocytogenes* ATCC19117 by Agar well Diffusion Assay



A: cell-free supernatant of *Lactobacillus curvatus* KNOUC4148
B: cell-free supernatant of *Lactobacillus curvatus* KNOUC4148 after heating at 100℃ for 30min

Fig.5 SDS-PAGE and Antimicrobial Activity of the Cell-free Supernatant of *Lactobacillus curvatus* KNOUC4148



A. Gel stained with Coomasssie Brilliant Blue G250.
1. molecular weight standards (broad range, BIO-RAD #161-031)
2. cell free supernatant of *Lactobacillus curvatus* KNOUC4148
3. molecular weight standards (polypeptide SDS-PAGE standards, BIO-RAD #161-0326)
B. Gel overlaid with Muller Hinton agar containing indicator organism, *Listeria monocytogenes*. Inhibition zone formed by the antimicrobial substance in cell free supernatant of *Lactobacillus curvatus* KNOUC4148 is indicated by an arrow

The bacteriocins of *Lb. plantarum* (Hernnadez *et al.,* 2005) and *Lb.* sp.(Vinod *et al.,* 2006) lost 25% and 60% of antimicrobial activity in 30 min and 20 min respectively at 100℃, and both of them were inactivated completely by autoclaving at 121℃. The antimicrobial substance of isolate KNOUC4148 did not lost its activity at the treatment at pH 4 to 8 for 1 h at 37℃(Table 3), showing that acidic and neutral environment is safe for the antimicrobial substance of strain KNOUC4148. Hydrolysis of antimicrobial substance produced by isolate KNOUC4148 in cell free culture supernatant with protease K and trypsin extinguished all the antimicrobial activity(Table 3), indicating that the antimicrobial substance is a proteinaceous one and it would be a bacteriocin. Proteolytic hydrolysis inactivated the bacteriocin of *Lb. curvatus* SE1(Kim *et al.,* 1998) and pediocins PA-1(Albano *et al.,* 2007). As in Table 3, α-Amylase hydrolysis inactivated the antimicrobial substance of isolate KNOUC4148, suggesting that amylolytic moiety of the antimicrobial substance may have an essential function on inhibitory activity. Bacteriocin of *Lb. curvatus* SE1 was not affected by α-amylase, but lost its antimicrobial activity by glucoamylase hydrolysis(Kim *et al.,* 1998).

Confirmation of Antimicrobial Substance on SDS-PAGE Gel

Cell free culture supernatant of isolate KNOUC4148 was analyzed on SDS-PAGE to confirm the existence of antimicrobial proteineous substance. The substance showed antimicrobial activity to *Listeria monocytogenes* at the region around 7-18 kDa on SDS-PAGE gel (Figure 5). The gel stained by Coomassie brilliant blue showed

main protein band at the same region with antimicrobial band. The Mw of bacteriocins produced by two strains of *Lb. curvatus* were differently reported to be 4.07 kDa(Garver and Muriana, 1994) and 6.257 kDa (Mohankumar and Arumugam, 2012).

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