Plantaricin W producer : *Lactobacillus plantarum* SS7 isolated from Isan-sausage (traditional Thai fermented meat-rice sausage)

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Abstract— **Strain SS7, isolated from Isan sausage (traditional Thai fermented meat-rice sausage) obtained from a traditional Thai fermented meat producer in Bangkok, was identified as *Lactobacillus plantarum* based on morphology, PCR and 16S rDNA sequencing. The strain revealed to produce bacteriocins which active against mostly gram positive bacteria such as lactic acid bacteria, *Bacillus coagulans*, *Enterococcus faecalis*, *Micrococcus luteus* and *Listeria innocua*. The bacteriocins produced by this strain were sensitive to proteolytic enzymes and heat stable under acidic condition. Identification of these purified bacteriocins revealed that the bacteriocins produced by strain SS7 belonged to 2-peptide bactriocins which related to two-peptide lantibiotics known as plantaricin W α (molecular mass of 3,223 Da) and plantaricin W β (molecular mass of 3,099 Da). Thus, this SS7 strain and its bacteriocins have potential for application as starter culture and food preservative, respectively, especially for the safety production of Isan sausage.**

Keywords— **plantaricin W, *Lactobacillus plantarum*, Traditional Thai fermented meat-rice sausage, Isan sausage**

I. INTRODUCTION

Lactic acid bacteria (LAB) are known for their production of antimicrobial compounds, including bacteriocins which are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (De Vuyst and Vandamme, 1994; Nes and Johnsborg, 2004). Their bactericidal mechanism vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (De Vuyst and Vandamme, 1994; James et al., 1991). A number of bacteriocins have been described for *Lactobacillus plantarum* isolated from various fermented products such as meat products, beverages and other food sources (Omar et al., 2006; Powell et al., 2007; Rekhif et al., 1995; Todorov et al., 2009) including traditional Thai fermented fish (Swetwiwathana et al., 2011).

Isan sausage, a traditional thai fermented meat-rice sausage, is normally made of minced pork, cooked rice, cooked salt, garlic and food additives, mixed well and stuffed tightly in edible casing. The product is left to ferment at the room temperature for 2-3 days. The preparation of this indigenous fermented product generally depends on a spontaneous or chance inoculation by naturally occurring lactic acid bacteria (LAB) mostly in genera Lactobacillus, Pediococcus and Micrococcus and the use of starter cultures is rare. Although information on bacteriocins in the literature is extensive, but little information has emerged regarding fermented foods of Thai origin, especially this traditional Thai indigenous fermented product. Thus, this study is to confirm the detection and characterization of bacteriocins produced by *Lactobacillus plantarum* SS7 isolated from a traditional thai fermented meat-rice sausage produced by Suddhiluck Innofood Co.,Ltd one of the most well-known traditional Thai fermented meat producers located in Bangkok, Thailand.

II. MATERIALS AND METHODS

1. Bacterial strains, media and cultivation conditions

SS7, the bacteriocin-producer strain, was isolated from Isan sausage (traditional Thai indigenous fermented meat-rice sausage). The strain was pre-identified by Gram-stain under microscope and acceptable with the results from both of catalase test (negative) and cell morphology (rod shape). The strain revealed to produce bacteriocins which active against mostly gram positive bacteria such as lactic acid bacteria, *Bacillus* spp., *Micrococcus luteus* and *Listeria innocua*. The bacteriocins produced by this strain were also reported sensitive to proteolytic enzymes and heat stable under acidic condition (Mettamethaet al., 2011). *Lb. sakei* subsp. *sakei* JCM 1157 [T] used for the bacteriocin activity tests was propagated in MRS medium at 30[o] C for 24 h. The strain of *Lb. Plantarum* NF3 was stored at -80[o] C in MRS broth containing 15% (v/v) glycerol. Before use, the strain was cultivated twice for 24 h at 30[o] C in MRS broth.

2. Identification the suspected bacteriocin-producing strain SS7 by 16S rDNA sequence

Partial phenotypic characterization of strain SS7 was performed by firstly preparing overnight cultures in MRS broth. 2 ml of overnight culture was harvested by centrifugation. The cells were then suspended in 80 µl of TE buffer (50mM Tris, 50mM EDTA, pH 8). Lysis was initiated by the addition of 5 mg/ml lysozyme. After incubation at 30 ^{o}C for 30 min, the mixture was further provided with MagExtracter-Genome (TOYOBO) as specified by the manufacturer. 16S rDNA gene region of strain SKA, corresponding to positions 8 to 1510, was analyzed by PCR (Zendo et al., 2005) using primer 8UA (5'- AGAGTTTGATCCTGGCTCAG –3') and 1510B (5'- GTGAAGCTTACG GCTACCTTGTTACGACTT –3') based on primers described by Martinez-Murcia et al. (1995). PCR product was then purified by using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Purified PCR product was used for DNA sequencing (Macrogen, Seoul, Korea). The obtained DNA sequences were analyzed using GENETYX-WIN software (GENETYX, Tokyo, Japan). Database searches were performed using BLAST of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/ BLAST/).

3. Determination of the concentration of antimicrobial produced from SS7 compared to known bacteriocins (pediocin PA-1)

The study was conducted by inoculating 1% of overnight culture of the selected potent LAB strain and known bacteriocin-producers such as pediocin PA-1 from *P. pentosaceus* TISTR 536 in MRS broth, and culturing for 20-22 h at 30^{o}C. The cultures were then centrifuged at 2,700 x g for 10 min. The supernatant from each of cultures was adjusted to pH 6.5 with 5.0 N NaOH and then filter-sterilized with 0.20 µm pore-size polysulfone membrane (Cica, Tokyo). The cell-free supernatant was determined for antagonistic activity on overnight cultures of indicators (Table 1) as recommended by Swetwiwathana et al. (2009) using spot-on-lawn method according to Ennahar et al. (1999) and Mayr-Harting et al. (1972) .

4. Bacteriocins purification

The cell-free supernatant of 1 liters culture incubated at 30 ^{o}C of NF3 was purified by a four step procedures as described by Ennahar et al. (1999). The final sample containing the purified bacteriocins was dried by Speed-Vac rotary evaporator (Savant Instruments) and stored at –20^{o}C for molecular mass determination.

5. Mass spectrometric

The molecular masses of purified bacteriocins were determined using a Accu TOF spectrometer, model JMS-T100LC (Agilent Technologies, Germany).

III. RESULTS AND DISCUSSION

1. Identification of the suspected bacteriocin-producing strain SS7 by 16S rDNA sequence

The confirmation results from about 1,500 bp phenotypic characterization of SS7 strain concurred also to early results of strain identification from morphology study which shown to be gram positive, rod shape and catalase negative. It was revealed that SS7 showed 99% identity of their DNA sequences to *Lb. plantarum* (Fig. 1). Thus, the antagonistic produces of this strain were used for further studied.

2. Determination of the concentration of antimicrobial produced from SS7 compared to known bacteriocins

The concentration of antimicrobials produced by SS7 was determined in arbitary unit per milliliter (AU/ml) with 7 indicators and compared to those of pediocin PA-1 producer of *P. pentosaceus* TISTR 536 (Table 1). Cross-reaction of the produced among SS7 strain, pediocin PA-1 was also studied. The results implied that the activity spectra of SS7 was differed from pediocin PA-1 producer of TISTR 536, and the bacteriocin produced from these 2 strains showed no effect on each other. According to these results we feel realize that both strains can be used as co-culture as starter culture for safety production of various Thai traditional fermented meat products.

3. Bacteriocins purificatio and mass spectrometric of bacteriocins from NF3

The cell-free supernatant from 1 liters culture incubated at 30^{o} C of *Lb. plantarum* SS7 was purified by a four step procedures as described by Ennahar *et al.* (1999). It was revealed that the bacteriocins produced by this strain might be a family of two-peptide bacteriocins, due to the purified fractions of bacteriocins after reverse phase HPLC exhibited 2 sections of activity (Fig. 2). The first highest activity fraction was shown around 21-23 min of Reverse Phase HPLC profiles and the second highest activity was exhibited around 29-31 min. The molecular masses of these two purified bacteriocins fractions were estimated to 3,101.7 d (Fig. 3) and 3,226 d (Fig. 4) respectively by using a Accu-TOF spectrometer Model JMS-T100LC (Agilent Technologies, Germany) for molecular weight determination. When compared the molecular weight results of bacteriocins from these 2 fractions, it was revealed that the molecular weight from these 2 purified fractions was

concurred to plantaricin W from *Lb. plantarum* which has been reported as a new family of two-peptide lantibiotics (plantaricin W α is 3,223 d and plantaricin W β is 3,099 d) by Holo *et al.* (2001). Thus, this bacteriocin-producing strain is under further study for possibility of using as starter culture for Isan sausage production.

IV. CONCLUSIONS

Consequently, identification of about 1,500 base pairs of 16S rDNA sequences of the isolated from Isan sausage (SS7) is confirmed as *Lb. plantarum.* This strain can produce bacteriocin which identified as plantaricin W-like bacteriocins. Hence, this potent plantaricin W-like producer strain was selected for our further study aimed on the potential use as starter culture for safety traditional Thai fermented meat (Isan sausage) production.

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ID : Gb[KT852804.1] ***Lactobacillus plantarum*** **strain L5 16S ribosomal RNA gene. Partial sequence**
Length = 1352 Score = 967 bits (488), Expect = 0.0
Identities = 494/496 (99 %), Gaps = 0/496 (0 %) Strand = Plus/Minus

Figure 1 : 16S rDNA sequences result of bacteriocin-producing strain NF3 by database searches from NCBI

Table 1 : Antimicrobial spectrum of bacteriocin (AU/ml) from SS7 compared to pediocin PA-1 (TISTR 536) producers.

Indicator	TISTR536	SS7
P. pentosaceus JCM 5885	200	1,600
Lb. sakei subsp. *sakei* JCM 1157 ^{T}	6,400	1,600
Lc. lactis subsp. *lactis* ATCC 19835 ^{T}	0	400
Micrococcus luteus IFO 12708	0	200
Listeria innocua ATCC 33090 ^{T}	12,800	400
Enterococcus faecalis JCM 5803 ^{T}	800	400
B. coagulans JCM 2257 ^{T}	0	1,600
TISTR536	0	0
NF	0	0

ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Japan; JM, commercial strain from Toyobo, Osaka, Japan; IFO, Institute for Fermentation, Osaka, Japan.

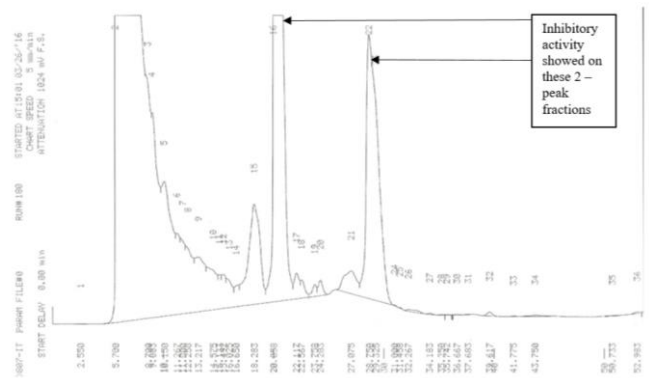

Figure 2 : Reverse Phase HPLC profiles of bacteriocins produced by SS7 (Active fraction showed at 21 and 29 min)
[Gradient condition : 0-5 min 20% acetonitrile (ACN); 6-36 min 20-60% ACN; 36-40 min 60-100 % ACN]

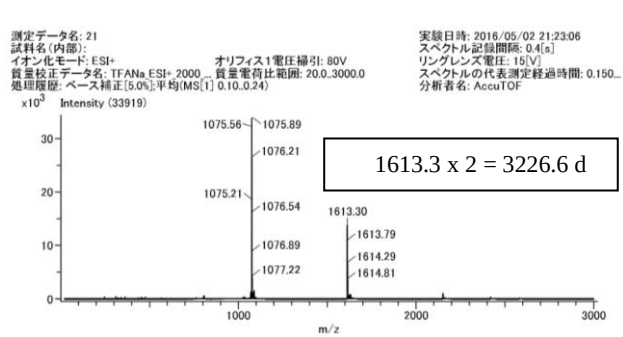

Figure 3 : Molecular mass of purified bacteriocin (first - 21 min) fraction from NF by Accu-TOF spectrometer Model JMS-T100LC

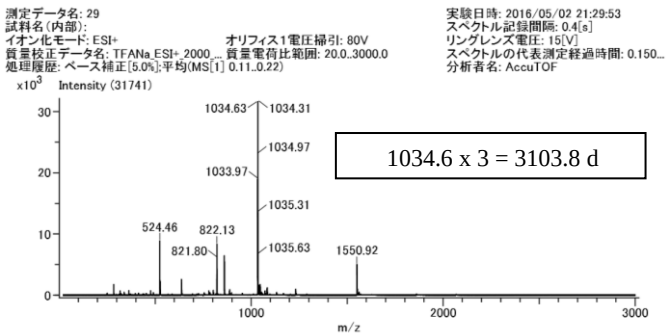

Figure 4 : Molecular mass of purified bacteriocin (second - 29 min) fraction from NF by Accu-TOF spectrometer Model JMS-T100LC