Screening of bacteriocin-producing lactic acid bacteria associated in Thai fermented meat-rice sausage

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Introduction

Lactic acid bacteria (LAB) are widely used as starter cultures for reliable and consistent acid production in various fermented foods. The inhibition of other microorganisms may also occur by the formation of various compounds, which produces during fermentation. Among the variety of these inhibitory compounds synthesized by these LAB, bacteriocins have been received much attention in the past decade (Ennahar et al., 1996; Gaenzle et al., 1997; Swetwiwathana, 2005). Thai fermented meat-rice sausage is a kind of traditional thai fermented meat, which 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 most important microorganisms during the spontaneous fermentation of this product belong to the LAB genera Lactobacillus, Pediococcus and Micrococcus. According to numerous reports on using LAB and bacteriocin-producing LAB as starter cultures to harm various pathogens in fermented foods (Hammes and Knauf, 1994; Ennahar et al., 1996) and traditional thai fermented meat such as Nham (Swetwiwathana et al., 1999; Swetwiwathana et al., 2005), thus, an attempt on finding the most potent bacteriocin-producing LAB strains from meat-rice sausage and use of these potent strains as starter cultures in order to improve the quality and safety during the fermentation of this product was studied and reported in this paper.

Materials and Methods

Isolation of bacteriocin-producing LAB from meat-rice sausage : A total of 150 strains were randomly isolated from the 15 samples of meat-rice sausage sold in Bangkok by spread plate technique on MRS agar (Oxoid) + 0.5 % calcium carbonate and incubated under micro-aerobic condition (candle jar) at 30° C for 48 h. Each strain with clear zone around colony was selected for detection of antagonistic activity. All strains were preculture overnight in MRS broth at 30° C before using in the step of screening test for their bacteriocins production. A special bacteriocin screening medium (BSM), which was developed on the basis of MRS medium Tichaczek *et al*. (1992) was used as bacteriocin screening medium for all isolates.

Determination of antagonistic activity : The agar spot assay (direct method) was performed essentially as described by Tichaczek *et al*. (1992). Evaluation of bacteriocin-producing strains was studied using the methods described by Tichaczek *et al*. (1992) and Ennahar *et al*.(1996) with nine indicators (Table 1). Antimicrobial producers were examined after 24 hours for zone of inhibition. The most potent strains, which showed the best inhibitory spectrum to the tested indicators (more than 5 indicators) and exhibited an inhibitory effect on food pathogens such as *E. coli*, *Lis. innocua*, *S. aureus* and *Salm. anatum*, were selected for further study.

Determination of the concentration of antimicrobial produced : 1% an overnight culture of the selected potent LAB strains was cultured in MRS for 20 h at 30°C. The cultures were then centrifuged at 2,700 x g for 10 min. The supernatant from each of cultures was adjusted to pH 7 with 5 N NaOH and then filter-sterilized with 0.2 µm pore-size polysulfone (Cica, Tokyo). The cell-free supernatant was determined an antagonistic activity by using spot on lown method as desbribed by Ennahar *et al*. (1996) with 14 indicators (Table 2), and antagonistic spectrum was compared to known bacteriocin producers.

Identification of the suspected bacteriocin-producing strains : The suspected bacteriocin-producing isolates were identified based on carbohydrate fermentation patterns by using API 50 CHL kit test (bioMorieux Vitek, Inc., Hazelwood, Mo.). Cell morphology of each isolate was studied with gram stains under microscope. The study of catalase test for each strain as recommended by Schillinger and Lücke (1989) was also performed.

Results and Discussion

2 of 150 strains were found to produce antagonistic compounds against more than 5 indicators (Table 1). Two strains of RS-49 and RS-54 showed their bactericidal board spectrum on more than 5 tested indicators and exerted the best bactericidal board spectrum on mostly gram positive indicators including food pathogens such as *S. aureus* and one gram negative indicator of *E. coli*. The results of catalase test, cell morphology and carbohydrate fermentation using API 50 CHL kit test of 2 selected isolates (Table 1) revealed that these 2 suspected bacteriocin-producers were belonged to *Lb. plantarum*. In order to confirm the coincidence of 'bacteriocin' definition from the produced of these 2 isolates, inhibitory spectrum profile of antagonistic produced of these isolated LAB was later compared to the spectrum of known pediocin PA-1 producer

(Swetwiwathana, 2005) and nisin A producer strain from NCDO (Table 2). It was found that an antagonistic produced by *Lb. plantarum* strains RS-49 and RS-54 exhibited the inhibitory spectrum profile related to pediocin PA-1 producer (TISTR536). Thus, these 2 *Lb. plantarum* might be the strains which produced bacteriocin related to pediocin PA-1. *Lb. plantarum*, which produced bacteriocin related to pediocin AcH/PA-1, has been found in cheese and reported by Ennahar et al. (1996). These 2 isolates, however, are currently under further study for the strain confirmation by 16S rDNA sequences, their bacteriocin purification and identification, and investigation to implement as starters for the best quality and safety in this traditional thai fermented meat production.

Table 1 : Pre-screening results of antagonistic substances produced by 2 of 150 strains isolated from meat-rice sausage using direct method

Indicator strain	**RS-49**	**RS-54**
Enterococcus faecalis JCM 5803 ^{T}	+	+
Escherichia coli JM 109	+	+
Lactobacillus sakei subsp. *sakei* JCM 1157 ^{T}	+	+
Lactococcus lactis subsp. *lactis* ATCC 19435 ^{T}	+	+
Listeria innocua ATCC 33090 ^{T}	++	++
Pediococcus pentosaceus JCM 5885	+	+
P. pentosaceus JCM 5890	-	-
Salmonella anatum WHO-BKK	-	-
Staphylococcus aureus ATCC 12600 ^{T}	+	+
Catalase test	-	-
Gram stain/cell morphology	gram positive/rod	gram positive/rod
API 50 CH test (% identity)	(99.9%)	(99.9%)

- = no inhibition/negative result, + = inhibition zone 1-5 mm., ++ = inhibition zone 6-10 mm.

Table 2 : Antimicrobial spectrum of MRS broth (cell-free supernatant) from isolated LAB

Indicator	**TISTR536**	**RS-49**	**RS-54**	**NCDO**
***P. pentosaceus* JCM 5885**	400	0	0	3,200
***Lb. plantarum* ATCC 14917 ^{T}**	6,400	200	200	1,600
***Lb. sakei subsp. sakei* JCM 1157 ^{T}**	6,400	400	400	25,600
***Lc. lactis subsp. lactis* ATCC 19435 ^{T}**	0	0	0	1,600
***Enterococcus faecium* TUA 1344L**	1,600	200	200	1,600
***Leuconostoc mesenteroides* JCM 6124 ^{T}**	1,600	0	0	3,200
***Micrococcus luteus* IFO 12708**	0	0	0	800
***Listeria innocua* ATCC 33090 ^{T}**	12,800	400	400	1,600
***Enterococcus faecalis* JCM 5803 ^{T}**	1,600	200	200	800
***Bacillus circulans* JCM 2504 ^{T}**	0	0	0	6,400
***B. coagulans* JCM 2257 ^{T}**	0	0	0	6,400
***B subtilis* JCM 1465 ^{T}**	0	0	0	3,200
***S. aureus* ATCC 12600^T**	0	0	0	1,600
***Escherichia coli* JM 109**	0	0	0	0

ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Japan; JM, commercial strain from Toyobo, Osaka, Japan; TUA, Tokyu University of Agriculture, Japan; IFO, Institute for Fermentation, Osaka, Japan; WHO-BKK, World Health Organization, Salmonella-Shigella Center, Bangkok, Thailand; TISTR 536, pediocin PA-1 producer from Swetwiwathana (2005); NCDO, nisin A producer from National Collection of Dairy Organisms, Reading, UK.

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