SCREENING OF BACTERIOCIN-PRODUCING BACTERIA ASSOCIATED IN NHAM (TRADITIONAL THAI FERMENTED MEAT)

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Background

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 [1-7)]. Nham is a kind of traditional thai fermented meat, which is normally made of minced pork, shredded cooked pig skin, cooked salt, garlic and food additives, mixed well and wrapped tightly in banana leaves or plastic bags. The product is left to ferment at the room temperature for 3-5 days. The most important microorganisms during the spontaneous fermentation of this product belong to the LAB genera Lactobacillus, Pediococcus and Micrococcus [8)]. According to numerous reports on the incidence of salmonellae in Nham [9, 10)], LAB and bacteriocin-producing LAB were applied as starter cultures to harm various pathogens in fermented foods [3, 5-7)] and this traditional thai fermented meat [9, 11)]. Thus, an attempt on finding the most potent bacteriocin-producing LAB strains from Nham and use of these potent strains as starter cultures could lead to improve the quality and safety in the production of Nham.

Objectives

The objective of the study is to isolate bacteriocin-producing bacteria and LAB with potential of use for increasing the microbiological safety of Nham. Besides, brief characterization of bacteriocins from the isolates is also reported in this paper.

Methods

Isolation of bacteriocin-producing bacteria from Nham: In order to search for bacteriocinogenic bacteria, a total of 300 strains were randomly isolated from the 15 samples of Nham sold in Bangkok, Chiangmai and Ubonratchathani by spread plate technique on MRS agar [12)] + 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. 9 strains of previously isolated lactic acid bacteria (LAB) from Nham obtained from Thailand Institute of Scientific and Technological Research (TISTR) (*Pediococcus* spp. TISTR 417, 419, 530, 536, 537 and *Lactobacillus* spp. TISTR 539, 540, 541, 543) were also used for detection of their antagonistic activity in this study. All strains were preculture overnight in MRS broth[12)] (Oxoid) at 30° C before using in the step of screening test for their bacteriocins production.

Indicator strains : Indicator strains, sources and culture conditions for each indicator are listed in Table 1.

Table 1 : Strains used in this study with their sources and culture conditions

Indicator strains (Source)[a]	Medium and culture condition [b]	Indicator strains (Source)[a]	Medium and culture condition [b]
Bacillus circulans (JCM 2504 ^{T})	TSBYE, 30° C, aerobic	*Lc. lactis* subsp. *cremoris* (TUA 1344L)	MRS, 30° C, anaerobic
B. coagulans (JCM 2257^{T})	TSBYE, 37° C, aerobic		
B. subtilis (JCM 1465 ^{T})	TSBYE, 30° C, aerobic	*Leuconostoc mesenteroides* subsp. *mesenteroides* (JCM 6124 ^{T})	MRS, 30° C, anaerobic
Enterococcus faecalis (JCM 5803 ^{T})	TSBYE, 37° C, aerobic		
Escherichia coli (JM 109)	TSBYE, 37° C, aerobic	*Listeria innocua* (ATCC 33090 ^{T})	TSBYE, 37° C, aerobic
Kokuria varians (LTH 1545)	TSBYE, 30° C, aerobic	*Lis. monocytogenes* (ATCC 19117)	TSBYE, 37° C, aerobic
Lactobacillus plantarum (ATCC 8014)	MRS, 30° C, anaerobic	*Micrococcus luteus* (IFO 12708)	TSBYE, 37° C, aerobic
		Pediococcus pentosaceus (JCM 5885)	MRS, 30° C, anaerobic
L. plantarum (ATCC 14917 ^{T})	MRS, 30° C, anaerobic	*P. pentosaceus* (JCM 5890 ^{T})	MRS, 30° C, anaerobic
L. sakei subsp. *sakei* (JCM 1157 ^{T})	MRS, 30° C, anaerobic	*Salmonella anatum* (WHO-BKK)	TSBYE, 37° C, aerobic
Lactococcus lactis subsp. *lactis* (ATCC 19435 ^{T})	MRS, 30° C, anaerobic	*Staphylococcus aureus* subsp. *aureus* (ATCC 12600 ^{T})	TSBYE, 37° C, aerobic
Lc. lactis subsp. *lactis* (NCDO 497)	MRS, 30° C, anaerobic	*S. carnosus* (LTH 2102)	TSBYE, 37° C, aerobic
Lc. lactis subsp. *lactis* IO-1 (JCM 7638)	MRS, 30° C, anaerobic		

[a] ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Japan; JM, commercial strain from Toyobo, Osaka, Japan; LTH, Lebensmitteltechnologie Hohenheim University, Stuttgart, Germany; TUA, Tokyu University of Agriculture, Japan; IFO, Institute for Fermentation, Osaka, Japan; WHO-BKK, World Health Organization, Salmonella-Shigella Center, Bangkok, Thailand. [b] MRS medium (Oxoid); TSBYE, Trypticase soy broth (Difco) + 0.6 % Yeast extract (Difco)

Bacteriocin screening medium : A special bacteriocin screening medium (BSM), which was developed on the basis of MRS medium [4)], was used as bacteriocin screening medium for all isolates.

Determination of antagonistic activity: The agar spot assay was performed essentially as described by Fleming *et al.* [13)]. Eva;uation of bacteriocin-producing strains was studied using the methods described by Tichaczek *et al.*[4)] and Ennahar *et al.*[6)] with twelves indicators (Table 2). 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. monocytogenes, S. aureus* and *Salm. anatum*, were selected for further study.

Determination of the concentration of antimicrobial produced, proteolytic enzyms sensitivity and heat treatment on antimicrobial activity : The study was conducted by inoculating 1 % an overnight culture of the selected potent LAB strains and culturing for 24 hours at 30° C. The cultures were then centrifuged at 2,700 x g for 10 minutes. The supernatant from each of cultures was adjusted to pH 7.0 with 5.0 N NaOH and then filter-sterilized with 0.20 μm pore-size polysulfone (Cica, Tokyo). The cell-free supernatant was determined for antagonistic activity by using spot on lown method as desbribed by Ennahar *et al.*[6)] and Mayr-Harting *et al.*[14)].

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 addition of catalase test for each strain as recommended by Schillinger and Lçcke[2)] was also performed in the study.

Results and Discussion

14 of 309 strains were found to produce antagonistic compounds against several indicators (Table 2). Six strains of N10, N39,N60, N100, N190 and TISTR536 showed their bactericidal board spectrum on more than 5 tested indicators. Among these potent strains, N100 and N190 were only the strains that exerted the best bactericidal board spectrum on mostly gram positive indicators including food pathogens such as *Lis. monocytogenes*, *S. aureus* and one gram negative indicator of *E. coli*. TISTR536 showed an inhibitory effect on the opportunistic food pathogens such as *E. coli* and *Lis. monocytogenes,* but the strain gave no inhibitory effect on *S. aureus* included *S. carnosus*, which is widely used as commercial starter cultures in various kind of European fermented meat sausages in order to enhance aroma and colour of meat products. N10, N39 and N60 inhibited only gram positive strains, but not any of food pathogen indicators. These 6 aforementioned strains were, thus, selected for further characterization. The results of catalase test, cell morphology and carbohydrate fermentation using API 50 CHL kit test of 6 selected isolates (Table 3), it can be concluded that we have now at least 3 groups of suspected bacteriocin-producers isolated from Nham. These 3 groups belong to *Lc. lactis* subsp. *lactis* (N100 and N190), *P. pentosaceus* (TISTR536) and unidentified gram positive tetrad cocci (N10, N39 and N60). These 3 groups of isolates, however, are currently under further identification step by 16S rDNA sequencing method.

In order to confirm the coincidence of 'bacteriocin' definition from the produced of 6 isolates, inhibitory spectrum profile of antagonistic produced from 3 groups of isolated LAB was later compared to the spectrum of known nisin A and nisin Z producers (Table 4). In addition, proteolytic enzymes and heat sensitivity of the produced from each strain had been performed (Table 5, 6). We found that various proteolytic enzymes and correlation between pH and heat treatment exerted inactivating effect on the produced from 6 isolates. The antagonistic produced by *Lc. lactis* strains N100 and N190 exhibited the inhibitory spectrum profile related to the both of known nisin producers. The produced of these 2 strains were sensitive to variuos proteolytic enzymes except pepsin and trypsin. Besides, the produced from both strains was sensitive to heat treatment at 121° C for 15 minutes under pH 7.0 and stable under low pH (3.0). With the coincidence of most results to the known nisin producers, it is assured that prior identify as *Lc. lactis* subsp. *lactis* of N100 and N190 are a group of bacteriocin-producers and their produces are related to nisin. The confirmation of the produced from other 2 groups of *P. pentosaceus* TISTR536 and unidentified strains of N10, N39

Table 2 : Preliminary screening results of antagonistic substances produced by 14 of 300 strains isolated from Nham against 12 indicators using colony spot on lawn

Indicator strain	New isolates (N)										LAB obtained from TISTR			
	10	17	19	39	44	53	60	75	100	190	419	530	536	543
Enterococcus faecalis	+	+	-	+	+	+	+	-	+	+	+	+	+	+
Escherichia coli	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Kocuria varians	+	-	-	+	-	-	+	-	+	+	-	-	+	+
Lactobacillus sakei subsp. *sakei*	+++	-	-	+++	-	-	+++	-	+++	+++	-	-	+	-
Lactococcus lactis subsp. *lactis*	+	-	-	+	-	-	+	-	-	-	-	-	-	-
Listeria innocua	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Lis monocytogenes	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Pediococcus pentosaceus	+	+	+	+	+	+	+	+	+++	+++	++	+	+	+
P. pentosaceus	+	+	+	+	+	+	+	+	++	++	-	-	+	+
Salmonella anatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus subsp. *aureus*	-	-	-	-	-	-	-	-	++	++	-	-	-	-
S. carnosus	++	-	-	++	-	-	++	-	+++	+++	-	-	-	-

- = no inhibition, + = inhibition zone 1-5 mm., ++ = inhibition zone 6-10 mm., +++ = inhibition zone > 10 mm.

Table 3 : Catalase test, morphology and carbohydrate fermentation (API 50 CHL) of potent strains

Test	N10	N39	N60	N100	N190	TISTR536
Catalase	+	+	+	-	-	-
Cell - morphology	tetrad cocci	tetrad cocci	tetrad cocci	short rod	short rod	tetrad cocci
Gram stain	+	+	+	+	+	+
API 50CHL	?	?	?	*Lc. lactis*	*Lc. lactis*	*P. pentosaceus*

+ = positive result, - + negative result, ? = unidentified

Table 4 : Inhibitory spectrum of antagonistic produced from isolated LAB in Nham (AU/ml)

Indicator strains (Source)	N10	N39	N60	N100	N190	TISTR 536	NCDO 497	IO-1 JCM7638
B.s circulans (JCM 2504 T)	800	800	800	1,600	6,400	0	800	3,200
B. coagulans (JCM 2257T)	400	400	400	1,600	6,400	0	3,200	6,400
B. subtilis (JCM 1465 T)	200	200	200	800	800	0	200	800
Ent. faecalis (JCM 5803 T)	100	100	100	400	800	800	100	400
E. coli (JM 109)	0	0	0	0	0	0	0	0
K. varians (LTH 1545)	200	200	200	400	400	6,400	400	400
L. plantarum (ATCC 8014)	200	200	200	400	800	0	200	400
L. plantarum (ATCC 14917 T)	0	0	0	200	800	6,400	100	400
L. sakei subsp. *sakei* (JCM 1157 T)	3,200	1,600	1,600	3,200	6,400	6,400	6,400	12,800
Lc. lactis subsp. *lactis* (ATCC 19435 T)	100	100	100	100	200	0	100	200
Lc. lactis subsp. *cremoris* (TUA 1344L)	0	0	0	200	800	1,600	100	400
Leu. mesenteroides subsp. *mesenteroides* (JCM 6124 T)	800	800	800	800	1,600	1,600	800	1,600
Lis. innocua (ATCC 33090 T)	0	0	0	400	1,600	6,400	100	800
Lis. monocytogenes (ATCC 19117)	0	0	0	800	1,600	6,400	nt	nt
M. luteus (IFO 12708)	0	0	0	400	1,600	0	200	800
P. pentosaceus (JCM 5885)	0	0	0	200	800	400	200	400
P. pentosaceus (JCM 5890 T)	0	0	0	200	200	200	0	100
Salm. anatum (WHO-BKK)	0	0	0	0	0	0	nt	nt
S. aureus subsp. *aureus* (ATCC 12600 T)	0	0	0	200	400	0	100	200
S. carnosus (LTH 2102)	1,600	1,600	1,600	3,200	3,200	0	1,600	3,200
Nisin A producer (NCDO 497)	0	0	0	100	0	0	0	100
Nisin Z producer strain IO-1 (JCM 7638)	0	0	0	0	0	0	0	0
TISTR 536	0	0	0	400	400	0	100	400
N10	0	0	0	1,600	1,600	0	1,600	1,600
N39	0	0	0	800	1,600	0	1,600	1,600
N60	0	0	0	800	1,600	0	1,600	1,600
N100	0	0	0	0	0	0	0	0
N190	0	0	0	0	0	0	0	0

nt = not tested

Table 5 : Sensitivity in AU/ml of the antimicrobial compounds produced by the suspected bacteriocinogenic producing LAB from Nham to various enzymatic treatments

Enzyme/Treatment	Residual activity (AU/ml)					
	N10	N39	N60	N100	N190	TISTR536
Control pH 3.0	1,600	1,600	1,600	12,800	6,400	6,400
Control pH 7.0	1,600	1,600	1,600	12,800	6,400	6,400
Ficin pH 7.0	0	0	0	100	100	0
α-Chymotrypsin pH 7.0	0	0	0	1,600	800	0
Trypsin pH 7.0	0	0	0	12,800	6,400	0
Pepsin pH 3.0	0	0	0	12,800	6,400	400
Protease type XIII pH 3.0	200	200	100	800	400	200

L. sakei subsp. *sakei* JCM 1157T was used as an indicator strain for the remaining activity.

Table 6 : Sensitivity of the antimicrobial compounds produced by bacteriocinogenic strains from to heat treatments.

Strain	Control	pH 7.0		pH 3.0	
		100° C 0 min	121° C 15 min	100° C 10 min	121° C 15 min
N10	1,600	400	0	800	400
N39	1,600	400	0	800	400
N60	1,600	400	0	800	400
N100	12,800	800	0	6,400	1,600
N190	6,400	800	0	3,200	1,600
TISTR536	6,400	1,600	0	3,200	1,600

L. sakei subsp. *sakei* JCM 1157T was used as an indicator strain for the remaining activity.

and N60 was revealed the similar results in their proteinaceous nature and heat sensitivity. But the bactericidal spectrum of these 2 groups was exhibited more narrow spectrum than N100 and N190. In view of their produced being proteinaceous nature, narrow inhibitory spectrum only gram positive indicators and sensitive to heat treatment under pH 7.0, we are, however, confident that both of these 2 groups are also in the group of bacteriocin-producers. Due to the inhibitory produced which effect on an opportunistic food pathogen of *L. monocytogenes* and ineffective on collaborate meat starter of *S. carnosus*, prior identify as *P. pentosaceus* TISTR536 is in our interest for further application as starter in Nham. Nevertheless, all isolated LAB are currently under further study for the strain confirmation by 16S rDNA sequences, their bacteriocin purification and identification, and investigation to implement their application for the best quality and safety in Nham production.

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