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Antimicrobial Activity of Extremely Halophilic Archaea Isolated from Southern Thai Salt-Fermented Products and Solar Saltern of Pattani, Thailand

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Abstract: This research aimed to study the diversity and antimicrobial activity of culturable haloarchaea in soil samples of solar saltern in Pattani Province, Thailand and Southern Thai salt-fermented food. Seventy-seven extremely haloarchaea were isolated on Halophilic medium agar containing 25% NaCl at 37°C for 7-21 days. They were grouped by Amplified Ribosomal DNA Restriction Analysis (ARDRA) with two restriction enzymes, *RsaI* and *HindIII*. The ARDRA patterns illustrated 6 different Operation Taxonomic Units (OTUs). Partial 16S rRNA gene sequences (938 bp) of the representative of each OTUs were aligned with the GenBank database. The results showed that the representatives of OTU1, OTU2, OTU3, OTU4, OTU5, and OTU6 had 99-100% similarity *to Halobacterium salinarum*, 99-100% similarity to *Halostagnicola larsenii*, 99% similarity to *Natronococcus* sp., 99-100% similarity to *Haloferax alexandrinus*, 99-100% similarity to *Natrialba* sp. and 97% similarity to *Halococcus* sp., respectively. The antimicrobial activity testing of the 17 isolated haloarchaeal strains from solar saltern was performed against 5 tested strains of *Hbt. salinarum* and 1 strain of *Natrialba* sp. were isolated from fermented food. Seven (41.2%) were candidate halocin-producing strains. In addition, no phage activity was observed. The 14-day fermentation broth of *Natronococcus* sp. SS13 showed the highest antimicrobial activity against *Hbt. salinarum* PK08, BD07 and BD09 (>5,120 AU/ml).

Keywords: Halophilic archaea; antagonistic activity; salt-fermented product; solar saltern

1. Introduction

Halophilic archaea (haloarchaea) are the members of the family *Halobacteriaceae*, order *Halobacteriales*, class *Halobacteria*, phylum *Euryarchaeota* in the domain *Archaea*. Halobacteriaceae, including at least 51 genera, have been validly characterized, reflecting their considerable ecophysiological diversity [1,2]. They require at least 1.5 M NaCl (9% w/v) for growth but grow optimally in 2.5 (15% w/v) to 5 M NaCl (30% w/v), and lower concentrations of salinity generally cause cell lysis [3]. Haloarchaea accumulates salts, mainly KCl, to adapt the entire intracellular machinery to function in the presence of high salts. Moreover, they can produce compatible organic osmotic solutes to maintain the concentration of ions inside and outside the cell to keep themselves intact and

remain alive in the hypersaline environment and salt-saturated ecosystem [4]. Several studies focusing on the microbial diversity in these ecosystems using culture-dependent or culture-independent approaches have shown that haloarchaea are the dominant colonizers [5,6]. When salinity reaches values close to saturation of NaCl, some archaeal populations become predominant to the exclusion of others. An important factor that may provide haloarchaea with a competitive advantage in competing for nutrients and other resources is the production of gene-encoded antimicrobial peptides or halocin proteins [7].

Halocins are either peptide (<10 kDa, microhalocins) or protein (>10 kDa) antibiotics. They have been reported to generally kill the indicator organisms by cell swelling followed by cell lysis [8]. Several previous studies have screened halocin-producing microorganisms from hypersaline environments [5,9,10]. Halocins have been reported to be produced among the Halobacteriaceae by species of *Haloferax*, *Haloterrigena*, *Natrinema,* and *Halobacterium* [11]. Moreover, the unique character of haloarchaea and their enzymes enabling them to sustain catalytic activity in hypersaline environments make them attractive resources for use in various industrial conditions. Several applications of haloarchaea and their value-added molecules have been characterized, i.e., halophilic and thermostable enzymes [12], bacteriorhodopsin [13], biodegradable polymers [14], bioremediation of polluted hypersaline environment [15], salt-fermented food [16] and carotenoid production [17].

Solar salterns, consisting of a series of shallow interconnected ponds filled with natural water from the sea, have been used for sea salt production. In Thailand, sea salts were produced by evaporating seawater and used in fermented food, especially fish sauce, fermented fish, salted fish, and pickled mussels. These products are rich in nutrients, particularly amino acids, and contain a high NaCl concentration, allowing several halophilic microorganisms to thrive. Some investigations of extremely halophilic archaea in Thai fermented food have been reported [18-21], but the haloarchaeal communities inhabiting the solar saltern of Southern Thailand have never been examined. Several studies have isolated and identified the strains of halophilic archaea from various fermented fish products. New species of halophilic archaea have been proposed, such as *Halobacterium piscisalsi* [18], *Natrinema gari* [19], *Haloarcula salaria*, *Haloarcular tradensis* [20], and *Halococcus thailandensis* [21]. However, the potential of such strains to produce halocins has also never been reported. Protein antibiotics can potentially preserve agents in the food and leather industries and control infectious bacteria. Hence, there is great interest in isolating potential proteinaceous bioactive substances. Therefore, in this study, members of the family *Halobacteriaceae* isolated from several Southern Thai salt-fermented products and the solar saltern soil of Pattani Province, Thailand, were screened to produce antimicrobial substances. For the first time, we also report members of the genera *Halostagnicola* and *Natronococcus* as producers of growth-inhibitory substances.

2. Materials and Methods

2.1 Isolation of extremely halophilic archaea

Extremely halophilic archaea were isolated from various Southern Thai traditional salt-fermented food products such as *budu* (Southern Thai fermented fish sauce), *pla-kem* (dried salted fish), *jing-jung* (fermented anchovy) and *hoi-dong* (pickled mussel), and solar saltern soils located in Pattani Province (Latitude 6° 53' 22.4592" N Longitude 101°16' 41.2795" E), Thailand. Samples were diluted at 1:10 in 25% NaCl. One-hundred microliters of diluted samples were plated on halophilic medium (HM) agar containing (l^{-1}) 250 g NaCl, 5 g casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_2O$, 0.036 g $FeCl_4.4H_2O$, 0.00036 $MnCl_2.4H_2O$, and 20 g agar (pH 7.5). The plates were incubated at 37 °C for 14-21 days. Representative colonies were transferred to fresh HM agar to obtain a pure culture.

2.2 Amplified ribosomal DNA restriction analysis

Genomic DNA was extracted from log-phase cells by lysed in purified water. Amplification of 16S rRNA gene fragment using archaeal primers set, 21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and 958R (5′-YCCGGCGTTGAMTCCAATT- 3′) as previously described [22]. Each 50 µl reaction PCR mixture contained 5 µl of 10x PCR buffer, 1 µl of dNTP (10 µM each), 1 µl of each primer (10 µM), 0.25 µl (1.25 U) *Taq* polymerase,

and 1 µl of template DNA. After a denaturation step of 5 min at 94 °C, amplification reactions were performed with 30 cycles of denaturation (1 min, 94 °C), primer annealing (1 min, 55 °C), and primer extension (1 min, 72 °C) with a final extension step at 72 °C for 7 min. The amplified DNA fragment (938 bp) was single-digested with *RsaI* and *HindIII* restriction endonucleases (Fermentas, Promega). Digestion reactions were performed in 25 µl containing 2 µl amplified 16S rDNA of the strains following protocol. The final concentration of restriction digestion mixture containing 1X appropriate buffer, 20-unit restriction enzyme, and make up the final volume with PCR grade water, restriction digestion was carried out at 37 °C for 2 hours, followed by enzyme inactivation at 65 °C for 30 minutes. The fragments from digested PCR products were analyzed in 1.5% agarose gel electrophoresis. PCR products were purified using an E.Z.N.A cycle pure kit and sequenced using primer 21F and 958R by the Macrogen sequencing facility (Macrogen Inc., Korea).

2.3 Antagonistic activity assay

To screen the antimicrobial activity of haloarchaeal strains, the antagonistic activity was carried out by using the agar double-layer diffusion assay. Briefly, 7 ml of melted soft HM agar (7.5 g/L agar maintained at 50 °C) was mixed with 10 µl of exponential phase culture of the target strain (OD_{600} = 0.4-0.6) and poured over HM agar (15 g/l). The target strains were BD06, BD07, BD09, PK08, JJ02, and HD07. Upon solidification, 5 µl of exponential phase culture of the potentially producing strain was spotted on the top agar. Incubation was carried out at 37 °C for 7 days until a homogenous microbial lawn and inhibition halos were observed.

2.4 Antimicrobial activity (Halocin activity) assay

The halocin activity was performed using the agar well diffusion assay as described previously [23] with some modifications. The selected bacterial strain with halocin activity was grown in 10 ml halophilic medium at 37 °C for 5 days. After growth, the culture obtained was centrifuged at 10,000 rpm at 4 °C for 15 min. Cells were harvested, washed with brine solution (25% NaCl), and resuspended in the brine solution. The cell suspension concentration was adjusted to 0.2 OD at 600 nm and used as inoculum at a 2% (v/v) level. 100 ml of halophilic medium in a 250 ml conical flask was inoculated with the prepared inoculums and incubated for 14 days. The bacterial-free supernatant was used for halocin assay after centrifugation (10,000 rpm for 10 min) of the culture broth. The halocin activity was checked by the agar well diffusion method. The tested strains (according to the result from 2.3) were grown until the OD reached 0.3 at 600 nm, and 200 µl of the tested strain was mixed with 20 ml of halophilic medium containing half-strength (0.75%) agar and overlaid on halophilic agar plates containing 1.5% agar. The halocin activity was checked by adding 50 µl of cell-free supernatant of selected producing strains into the well (0.5 cm diameter) made on the plate containing the top agar and performing the halocin assay after incubation for 7 days. The halocin activity was determined using serial twofold critical endpoint dilutions to extinction and expressed as arbitrary units (AU), defined as the reciprocal of the first dilution at all traces of inhibitory activity disappear. The two-fold dilution ratio of halocin follows a geometric progression where the halocin activity can be calculated by Arbitrary unit (AU/ml) = (1,000/A)/B. Where "A" is the volume of supernatant and "B" is the highest dilution of the supernatant at which inhibitory activity still appears.

2.5 Detection of phage activity

To determine the phage activity, a fragment of agar was cut from the halo zone of inhibition of the sensitive strain and added to 100 ml of HM inoculated with 100 µl of the culture of the sensitive strain. The culture (with and without agar fragments) was then incubated at 37°C for 7-14 days. The growth was determined by measuring OD_{600} (compared to the growth without the agar fragment). Plague assay was performed by inoculating crude culture supernatant with sensitive strain in soft agar medium as described above.

3. Results and Discussion

This study isolated 77 halophilic archaeal strains from Southern Thai traditional salt-fermented food products and solar saltern soils. Amongst 77 isolates, 20, 15, 15, 10, and 17 isolates were from *budu* (designated as BD01-BD20), *pla-kem* (PK01-PK15), *jing-jung* (JJ01-JJ15), *hoi-dong* (HD01-HD10) and solar saltern soil

(SS01-SS17), respectively. Colonies of all selected isolates were circular with entire edges, pink and orange to red, except all 15 HD isolates, which were colorless. All isolates were catalase-positive, oxidase-positive, gram-negative, aerobic rods with optimum growth at 37-42 °C, pH 7-8 in the medium containing 20-25% salt concentration. When the ARDRA method was used to group all haloarchaeal isolates, the restriction profiles revealed significant genetic differences among the strains, confirming that the isolates represented at least 6 different operational taxonomic units (OTUs) groups from 77 isolated haloarchaea. *RsaI* digestion classified all isolates into 4 restriction patterns, while *HindIII* digestion generated 3 patterns (Figure 1).

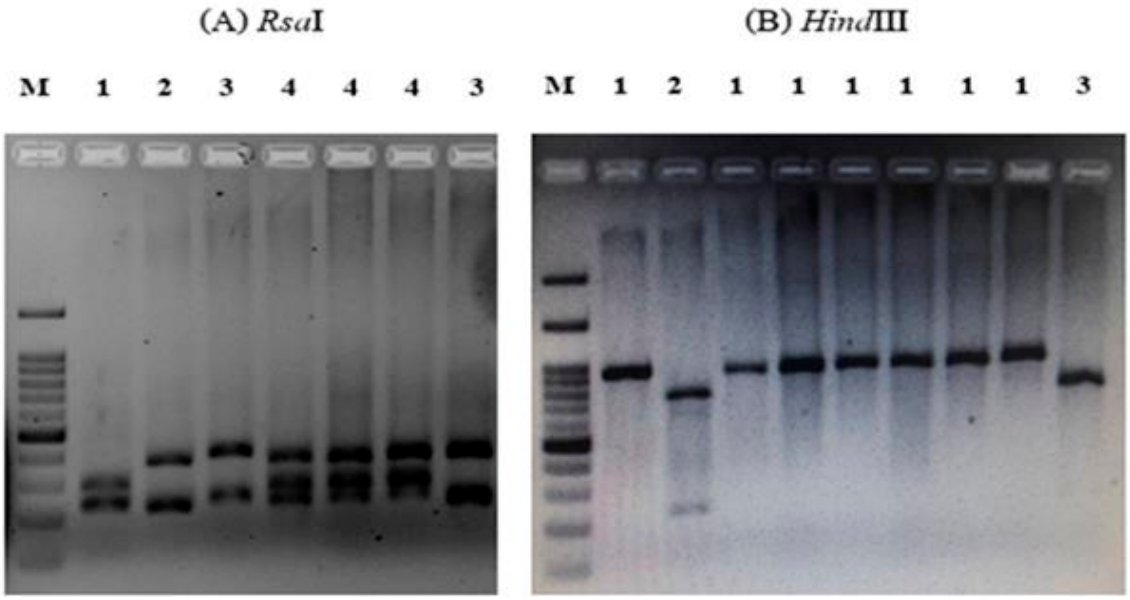


Figure 1. Four and three restriction fragment patterns of 938 amplified DNA of haloarchaeal strains digested with *RsaI* (A) and *HindIII* (B), respectively. M; 100 bp DNA ladder, 1-4; restriction patterns.

When combined *RsaI* with *HindIII* digested patterns, 6 OTUs were classified. The isolate belonging to OTU1 was found in all samples, while the isolate belonging to OTU2, 3, and 4 were found in solar salterns. Partial 16S rRNA gene sequencing was performed for at least 2 representatives of each OTUs and was analyzed using the BLAST algorithm. The representative strains of each OTUs were identified to the different genera, which were *Halobacterium* (OTU1), *Halostagnicola* (OTU2), *Natronococcus* (OTU3), *Haloferax* (OTU4), *Natrialba* (OTU5) and *Halococcus* (OTU6) by the similarity of 97-100% compared with the previously reported strains (Table 1).

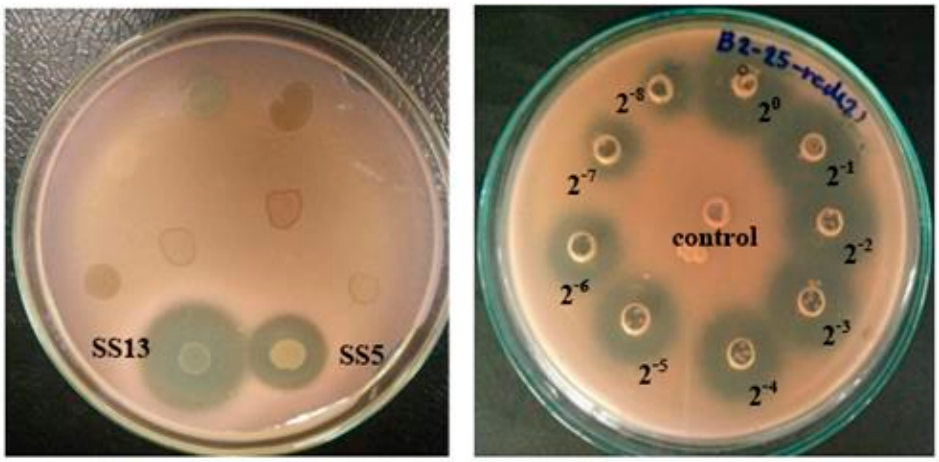
Together with the colony pigmentation and the requirement for high salt concentrations, these properties suggested that all strains might be members of the family *Halobacteriaceae*. The isolates revealed high similarity with the closest described species regarding the colony, cell morphologies, and physiological characteristics. Only 3 haloarchaeal genera (*Halobacterium*, *Halococcus,* and *Natrialba*) were isolated from salt-fermented food products, but all 6 genera were found in soil samples from the solar saltern of Pattani, Thailand. *Hbt. salinarum* seems to be the most abundant haloarchaea in all salt-fermented food products except *hoi-dong* (pickle mussel), in which Natrialba predominates. ARDRA technique was applied for several archaeal diversity studies, such as in superficial hypersaline sediments of solar salterns in Tunisia [24] and saltpan sediment in India [25]. Our results showed that *RsaI* and *HindIII* could be helpful for the primary grouping of haloarchaea isolated from a solar saltern in Thailand, as they can classify at least 6 restriction patterns.

All 17 (SS01-17) halophilic archaeal strains from solar saltern were tested for their antagonistic ability against 6 tested halophilic microorganisms (BD06, BD07, BD09, JJ02, PK08, and HD07), which were isolated from fermented food samples. Among those, 7 strains (41.2%) showed antagonistic activity against at least 2 of 6 testing haloarchaeal strains (Table 2). SS01, SS05, SS10, and SS16 exhibited antagonistic activity against

only two target strains, while three other strains (SS03, SS13, and SS14) could inhibit the growth of several testing isolates. Different degrees of activity were observed. The genus *Natronococcus* (SS03 and SS13) were the most active and might be the best producer of antimicrobial compounds (Figure 2, Table 2-3). *Hbt. salinarum* PK08 was the most sensitive. It showed sensitivity to all 7 producing strains.

Table 1. The results and maximum identification percentage were obtained from the NCBI nucleotide sequencing program and 6 OTU groups of the isolated haloarchaeal strains.

OTU group (*Rsa*I-*Hind*III)	Strains	Representing strain	Identification/accession no.	%Similarity
OTU 1	JJ01-15	JJ02	*Halobacterium salinarum* KP751341.1	99
(1-1)	PK01-15	PK08	*Halobacterium salinarum* KR611163.1	99
	BD01-19	BD06	*Halobacterium salinarum* KR611163.1	100
	HD10	BD07	*Halobacterium salinarum* NR113428.1	99
	SS07-09, 11-12	BD09	*Halobacterium salinarum* NR113428.1	99
	SS14-16	SS14	*Halobacterium salinarum* NR113428.1	99
		SS16	*Halobacterium salinarum* NR113428.1	99
OTU 2	SS01-02, 10	SS01	*Halostagnicola larsenii* KP117067.1	99
(2-1)		SS10	*Halostagnicola larsenii* NR113506.1	100
OTU 3	SS03, 13	SS03	*Natronococcus* sp. JX481739.1	99
(3-2)		SS13	*Natronococcus* sp. DQ373054.1	99
OTU 4	SS04-05	SS04	*Haloferax alexandrinus* NR113438.1	99
(4-1)		SS05	*Haloferax alexandrinus* NR113438.1	100
OTU 5	HD01-09	HD02	*Natrialba taiwanensis* AB663459.1	99
(3-1)	SS17	HD03	*Natrialba taiwanensis* AB663459.1	99
		HD07	*Natrialba aegyptia* JX481742.1	99
		SS17	*Natrialba aegyptia* JX481742.1	99
OTU 6	BD20	BD20	*Halococcus thailandensis* EU984192.1	97
(1-3)	SS06	SS06	*Halococcus* sp. AB904834.1	97



(A) (B)

Figure 2. The antagonistic activity of *Haloferax* SS05 and *Natronococcus* SS13 against sensitive strain *Hbt. salinarum* BD07 (A) and antimicrobial activity of the supernatant of strain *Natronococcus* SS13 against *Hbt. salinarum* BD07 (B)

Table 2. Antagonistic activity of haloarchaea from solar saltern (SS) against at least 1 strain of tested haloarchaea from fermented food products (BD, PK, JJ, and HD)

Producer strains	Inhibition zone of target strains (mm)					
	BD06	**BD07**	**BD09**	**PK08**	**JJ02**	**HD07**
SS01	-	-	13	15	-	-
SS03	18	-	28	28	15	-
SS05	-	20	-	14	-	-
SS10	-	-	-	12	12	-
SS13	-	28	27	26	32	-
SS14	12	-	12	11	-	-
SS16	-	-	12	12	-	-

Table 3. Antimicrobial activity of 7 candidate halocin-producing strains measured in arbitrary unit (AU)/ml

Producer strain	Target strain	Arbitrary Units (AU)/ml
Halostagnicola SS01	*Hbt. salinarum* PK08	640
Natronococcus SS03	*Hbt. salinarum* PK08	1,280
	Hbt. salinarum BD06	1,280
	Hbt. salinarum BD09	≥ 5,120
Haloferax SS05	*Hbt. salinarum* BD07	≥ 5,120
Halostagnicola SS10	*Hbt. salinarum* JJ02	80
	Hbt. salinarum PK08	160
Natronococcus SS13	*Hbt. salinarum* PK08	≥ 5,120
	Hbt. salinarum BD07	≥ 5,120
	Hbt. salinarum BD09	≥ 5,120
Halobacterium SS14	*Hbt. salinarum* PK08	20
	Hbt. salinarum BD09	40
Halobacterium SS16	*Hbt. salinarum* PK08	40
	Hbt. salinarum BD09	80

The halophilic archaeal strains with antagonistic activity against other microorganisms were selected for further halocin activity assay observation. The culture supernatants of those producing strains were determined for their activities in arbitrary units (AU)/ml against their sensitive strains. SS01, SS03, SS05, and SS13 showed inhibitory activity against *Hbt. salinarum* PK08 of 640, 1,280, >5,120 and >5,120 AU/ml, respectively. SS05 and SS13 showed the equal highest inhibitory activity against *Hbt. salinarum* BD07 of >5120 AU/ml. From the inhibitory assay in AU/ml, the culture supernatant of the strain of the genus *Natronococcus* (SS03 and SS13) and *Haloferax* (SS05) was the most active. It might be the strongest producer of antimicrobial compounds.

The diversity of haloarchaeal strains from the solar saltern of Pattani Province, Thailand, and the potential antimicrobial production of those strains have not yet been reported. In this study, 17 haloarchaeal strains were isolated and were examined for their ability to exert antimicrobial activity against 6 testing haloarchaeal strains from several salt-fermented foods. This work isolated 19 strains of *Halobacterium* and 1 strain of *Halococcus* from *budu*, the Southern Thai fermented fish sauce. Previous studies consistently reported that several haloarchaeal genera were isolated from Thai fish sauce products, including *Halobacterium*, *Natrinema, Haloarcula,* and *Halococcus* [18-21]. Furthermore, non-pigmented *Natrialba* spp. were the major haloarchaea in hoi-dong (salt-fermented mussel), consistent with the previous report that *Natrialba aegyptia* was isolated using culture-dependent methods of *jeotgal*, Korean fermented fish and shellfish [26]. For the diversity of haloarchaea in the solar saltern of Pattani, 6 genera were identified, including *Halobacterium, Halococcus*, *Halostagnicola, Haloferax*, *Natronococcus,* and *Natrialba*. This result correlates with several studies that reported that haloarchaea had been isolated from different hypersaline habitats, especially solar saltern.

Culture-based assessment of archaeal diversity in solar saltern from other regions of the world has been identified, which *Haloarcula, Halobacterium, Halorubrum,* and *Haloferax* as the main genera [27]. Atanasova and coworkers have isolated 11 haloarchaeal genera from the solar saltern of Samutsakorn Province, another solar saltern of Central Thailand, including *Halorubrum, Halolamina, Halobacterium, Halobellus, Haloarcula, Halogeometricum, Haloterrigena, Halogranum, Haloferax, Halosarcina* and *Natrinema* [28-29].

Seven strains exhibiting antimicrobial activity belong to 4 genera (2 *Halobacterium,* 2 *Halostagnicola,* 1 *Haloferax*, and 2 *Natronococcus*). It has been reported in previous studies that a significant fraction of haloarchaea inhabiting hypersaline areas produce antimicrobial agents [30]. A previous study reported the antagonistic interactions among halophilic archaeal isolates of distant geographical sites [31]. Several genera of halophilic archaea, including *Halorubrum, Haloferax, Haloplanus, Haloarcula, Halogranum, Halobacterium, Halosarcina, Halogeometricum* [32], *Natrinema, Halopiger* [9] and *Haloterigena* [5] are halocin-producers. For the first time, in the present work, we reported that genera *Natronococcus* and *Halostagnicola* had also been shown to be candidate halocin producers.

Antimicrobial activities produced by haloarchaeal strains may be due to agents of various nature-lytic viruses or antimicrobial peptides/proteins that either may be secreted or remain bound to the wall of the producing cells [5]. Multiple dilutions of producer strain culture supernatants were applied on indicator lawns to confirm inhibition was not a result of phage infection. No plaques were observed in a plaque assay using crude or diluted culture supernatant inoculated together with sensitive strain in a soft agar medium, demonstrating the absence of the virus. Halophilic archaea were the first archaea domain members found to produce archaeocin, bacteriocin-like proteins, also known as halocin. Halocins were initially discovered during a survey of antagonistic interactions among different members of the class *Halobacteria* [31]. At least 11 halocins have been reported, including HA1, HA3, A4, H1, H4, H6/H7, R1, Sech7A, SH10, S8, and C8 [7,8,23,30,32-35]. Among these, only 3 genes, *halH4*, *halS8*, and *halC8* coding halocin H4, S8, and C8, have been identified and described [36-38]. However, all our producing strains gave negative results for PCR using *halH4*, *halS8*, and *halC8* specific primers (data not shown). The halocin will be purified and characterized in a future study to confirm that the antagonistic activity of 7 candidate halocin-producing strains was due to halocin production.

4. Conclusions

ARDRA analysis of 77 haloarchaeal strains was classified into 6 OTUs. According to the comparison of the 16S rDNA sequences, 17 representative strains were affiliated with six different genera, including *Halobacterium, Halococcus, Natrialba, Haloferax, Natronococcus,* and *Halostagnicola*. The first 3 genera were found in Southern Thai salt-fermented food products, while all 6 were found in solar saltern. Some isolates of the 4 genera; 2 isolates of *Halostagnicola* (SS01 and SS10), 1 isolate of *Haloferax* (SS05), 2 isolates of *Halobacterium* (SS14 and SS16), and 2 isolates of *Natronococcus* (SS03 and SS13) showed antagonistic activity against testing haloarchaea. Candidate halocin-producing strain *Natronococcus* sp. SS13 showed the highest antimicrobial activity against *Hbt. salinarum* PK08, BD07 and BD09.

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