CHAPTER V

CONCLUSION

In the course of investigation of halophilic bacteria presented in pla-ra, fifty seven bacterial isolates were characterized taxonomically. They were divided into nine groups based on their phenotypic characteristics and 16S rDNA sequence analyses of the representative isolates. Fifty five isolates were Gram-positive (Group I to VII), endospore-forming rod-shaped bacteria . Two isolates were Gram-negative rod-shaped beacteria (Group VIII and IX). The isolates in Group I (10 isolates), II (13 isolates), III (13 isolates), IV (1 isolate), V (10 isolates), VI (6 isolates), VIII (1 isolate) and IX(1 isolate) required NaCl for growth in contrast the isolates in group VII showed good growth in the absence of NaCl. However, Group VII isolates were tolerant in 10% NaCl medium. The results indicated that Group I, II, III, IV, V, VI, VIII and IX isolates were moderately halophilic bacteria and Group VII isolates were halotolerant. All Gram-positive rods contained *meso*-DAP as a diagnostic diamino acid in the cell wall peptidoglycan except Group VI contained L-Orn type. The predominant isoprenoid quinone found was menaquinone-7 (MK-7). The two Gram-negative bacteria in Group VIII and IX. contained ubiquinone-8 and ubiquinone-9 as predominant isoprenoid quinone, respectively.

Group I, II, III and IV isolates belonging to the Genus *Virgibacillus*. On the basis of 16S rDNA sequence analyses, the representative of Group I (MSK2-1), II (CHM1-4), III (TP3-3) and Group IV (MS3-4) were closely related to *V. dokdonensis* (99.8%), *V. halodenitrificans* (99.6%) and *V. marismortui* (99.6%), respectively. In addition, Group I, II and III isolates showed high DNA-DNA relatedness with theirs type strains. Thus, Group I, II and III isolates were identified as *V. dokdonensis*, *V. halodenitrificans* and *V. marismortui*, respectively. However, strain MS3-4 (Group IV) exhibited low 16S rDNA sequence similarity values with *Virgibacillus* species (93.6-96%). This strain should be the novel halophilic bacterium that be required further studies on the DNA-DNA hybridization with related *Virgibacillus* species.

Group V contained only one isolate, TP2-8. On the basis of 16S rDNA sequence analyses, TP2-8 was closely related *to G. saliphilus* YIM 91119^T (99.2%). Major cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, and anteiso-$C_{17:0}$. Polar lipids were contained diphosphatidyl glycerol, phosphatidylglycerol, and unidentified glycolipid. The DNA G+C content was 37.6 mol%. In addition, the low DNA-DNA relatedness to the type strains of *Gracilibacillus* species indicated that TP2-8 was a new species for which a name *Gracilibacillu. thailandensis* sp. nov. was proposed.

Group VI, the isolates in this group were divided into two groups, the first contained TPA3-2, H1-1, AG2-2 and AG1-3 and the other were BY1-1 and MS2-6. Based on the 16S rDNA sequence of representative isolate, TPA3-2 was closely related to the to *H. locisalis* MSS-155^T (99.5%), *H. trueperi* DSM 10404^T (99%), *H. faecis* IGA7-4^T (99%), and *H. dabanensis* D-8^T (99%). TPA3-2 showed DNA-DNA relatedness lower than 70% with H. locissalis KCTC 3788^T(=MSS-155^T) strain BY1-1 and MS2-6. They were the novel halophilic bacteria but their phenotypic characteristics, 16S rDNA sequence analyses and DNA-DNA hybridization are required for further studies with the related *Halobacillus* species.

Group VII, the isolates belonged to the Genus *Bacillus* based on 16S rDNA sequence analyses. The representative isolate N20-1 was closely related to *Bacillus vietnamensis* (99.6%), *B. aquimaris* (99.3%) and *B. marisflavi* (98.4%). In addition, the results of DNA-DNA hybridization could be separated them into two groups, isolates TP4-7and N20-1 that were closed to *Bacillus vietnamnensis,* and the remained eight isolates were the other group. Their phenotypic characteristics, 16S rDNA sequence analyses and DNA-DNA hybridization are required for further studies with the related *Bacillus* species.

Group VIII, ND1-1 was belonged the genus *Salinivibrio* based on 16S rDNA sequence. It was closely related to *S. costicola* subsp. *costcola* NCIMB 701^T (98.3%), *S. costicola* subsp. *alcaliphilus* DSM 16359^T (98.3%), *S. costicola* subsp. *vallismortis* DSM 8285^T (98.2%) and *S. proteolyticus* AF-2004^T (98.6%). Phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol were the predominantant polar lipids. DNA G+C content of ND1-1 was 49.0 mol%. DNA-DNA relatedness indicated that ND1-1 was new species of the genus *Salinivibrio* for which a name *Salinivibrio sianensis* sp. nov. was proposed.

Group XI, R5-7 exhibited sequence similarity values of 98.8% with *C. salexigens* KTCC 12941^T. Result of DNA-DNA hybridization revealed that the strain R5-7 isolate was exhibited relatively high levels of hybridization with *C. salexigens* KCTC 12941^T (98.7%). In addition, their phenotypic characteristics were similar to *C. salexigens* KCTC 12941^T. R5-7 isolate was identified as *C. salexigens*.

Among 57 isolates, Group VII (ND1-1) strain ND1-1 was selected for further study due to the novelty of the strain and high protease production. The moderately halophilic bacterium, strain ND1-1 produced extracellular protease at the middle of exponential phase. The maximum protease production of ND1-1 was at the beginning of stationary phase and was achieved when cultivated in a modified JCM no. 377 medium omitted casamino acids, containing 10% NaCl, at pH 8.0 and incubation at 37°C for 2 days. Under the optimal condition, crude protease produced by strain ND1-1 increased 6.25 times. The purified protease from ND1-1 was monomeric protein with the molecular mass of about 36-38 kDa. The enzyme had a maximal activity in the presence of 5% w/v NaCl, at pH 8.0 and at 55°C. Stability remained more than 50% in the presence of 5-30% w/v NaCl, pH 5.0-9.0 and at 30–55 °C. The enzyme was identified to be metallo-protease .

From the results mentioned above, the moderately halophilic bacteria, *Virgibacillus, Halobacillus, Gracilibacillus, Salinivibrio* and *Chromohalobacter* strains including the halotolerant *Bacillus* strains were distributed in many samples of pla-ra. They are the most likely source of enzymes and constitute a heterogeneous group of halophiles belonging to different genera. The isolation of halophiles able to produce extracellular enzymes will provide the possibility to have optimal activities at different salt concentrations. *Salinivibrio siamensis* ND1-1 produced a substantial level of extracellular proteolytic activity that was active in extreme conditions. The applications of this strain and the roles of the remained strains should be further study.