CHAPTER IV

RESULTS AND DISCUSSION

4.1 Bacterial isolation and source of samples

A total of 57 isolates were isolated from total 46 samples of pla-ra collected in Thailand (Table 4.1). The pH of the pla-ra samples ranges from 5.5-6.0.

Table 4.1 Location, isolate number, and number of isolate

Location of samples	Isolate no.	No. of isolate
Bangkhunsri, Bangkok	BKS1-1	1
Thaewast, Bangkok	BT3-1	1
Bangkok	AG1-3,AG2-2,	2
Chainat	CHN1-1, CHN2-2	2
Chiang Mai	CHM1-3, CHM1-4, CHM2-2	3
Ladkrabang, Bangkok	BKL1-1, BKL2-2, BKL4-2, BKL 5-3, BKL6-1	5
Lop Buri	LB3-2	1
Mahasarakhram	MSK1-1, MSK2-1, MSK3-1	3
Mahasarakhram	MS1-3, MS2-6, MS3-2,MS3-4	4
Nakhon Nayok	ND1-1, ND2-1, N20-1	3
Nakhon Ratchasima	RM1-1, RM2-1	2
Nakhon Sawan	NS1-7	1
Nonthaburi	TPA1-1,TPA1-3, TPA1-7,TPA2-1, TPA2-6, TPA2-8, TPA3-1,TPA3-3, TPA4-3, TPA4-4, TPA4-6, TPA4-7, HR1-1,TPA3-2	14
Phichit	PJ1-6	1
Prachinburi	PR2-1, PR2-2	2
Phra Nakhon Si Ayutthaya	AY1-2	1
Roi Et	R5-7	1
Samrong, Samut Prakarn	BSR4-1, BSR3-1, TSR2-1, TSR5-2	4
Samyan , Bangkok	TSY2-3	1
Sing Buri	SB2-2	1
Sukho Thai	BY1-1, BY2-4	2
Talaadthai market, Bangkok	TM4-3, PT5-1	2
Total		**57**

4.2 Identification and characterization of isolates

Fifty-seven isolates were identified based the phenotypic characteristics and the 16S rDNA sequence analyses of the representative strain in each of group. They were divided into nine groups, fifty five isolates in Group I to VII are Gram-positive rod-shaped bacteria. Two isolates in Group VIII and IX are Gram-negative rod-shaped bacteria. The isolates in Group I, II, III, IV, V, VI, VIII and IX required NaCl for growth therefore they grew in NaCl containing medium better than when there was no NaCl, in contrast group VII, showed better growth in the absence of NaCl. However, the isolates in Group VII were tolerant to 10% w/v NaCl (Kushner 1985). The results of chemotaxonomic characteristics and DNA-DNA relatedness were supported their identification.

4.2.1 Group I

Group I contained ten isolates, BKL4-2, BKL5-3, CHM2-2, CHN1-1, CHN2-2, LB3-2, MSK1-1, MSK2-1, MSK3-1 and TP2-1. Cell size was approximate 0.6-0.8 x 2.5-5 μm. Colonies were irregular, flat, milky white in colour (0.5 – 3.5 mm diameter) after 3 days of incubation at 37°C on JCM no. 377 medium. They are motile. Terminal and subterminal endospore were observed under microscopy. Catalase and oxidase activity were positive but negative for L-arginine dihydrolase urease, nitrate reduction and H_2S formation. They hydrolyzed aesculin, casein, gelatin, starch and Tween 80. They grew in NaCl 0 - 20% w/v and at pH 5.0 – 8.0. Optimum growth temperature was 30-37°C, but not grew at 15 or 55°C. They grew under aerobic and anaerobic condition. They produced acid produce from L-arabinose, cellobiose, fructose, galactose, D-glucose, glycerol, inulin, myo-inositol, lactose, maltose, mannitol, mannose, melezitose, ribose, salicin, sucrose, and D-xylose, but did not produce acid from melibiose, raffinose, rhamnose and trehalose.

They contained *meso*-diaminopimelic acid (*meso*-DAP) as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant quinone was menaquinone with seven isoprene units (MK-7). The DNA G-C content of MSK2-1 was 36.5 mol%. On the basis of 16S rDNA sequence and phylogenetic analyses, the representative isolate, MSK2-1 in Group I (1,491nt) was clustered within a clade of the genus *Virgibacillus* (Figure 4.2). It was closely related to *V. dokdonensis* (99.8%)

(Table4.6). Isolate MSK2-1, *V. dokdonensis* KCTC 3933^T and related isolates showed high DNA-DNA relatedness (89.1-100%) (Table4.2). The high values of DNA-DNA relatedness more than 70% (Wayne et al., 1987), concluded that Group I isolates were identified as *V. dokdonensis* (Yoon et al., 2005).

Table 4.2 DNA-DNA relatedness of the isolates in Group I and *V. dokdonensis* KCTC 3933^T.

Isolate	%DNA-DNA relatedness with labeled strains	
	MSK2-1	KCTC 3933^T
MSK2-1	100	95.8
BKL4-2	98.1	101.1
BKL5-3	103.7	108.3
CHM2-2	83.9	95.1
CHN1-1	85.0	105.1
CHN2-2	89.9	105.0
LB3-2	87.0	100.4
MSK1-1	87.5	100.9
MSK3-1	88.6	106.5
TP2-1	94.7	89.1
V. dokdonensis KCTC 3933^T	97.3	100

4.2.2 Group II

Group II contained 13 isolates, AY1-2, BKL1-1, BKL2-2, BKL6-1, CHM1-3, CHM1-4, TPA3-1, TP4-3, TPA2-6, TSY2-3, BSR3-1, PT5-1 and TSR2-1. Cell size was approximate 0.6-0.8 x 2.0-4.0 μm. Colonies were circular to slightly irregular, raised, cream brown in colour and 1-2 mm diameter after 3 days of incubation at 37°C on JCM no. 377 medium. They are motile. Terminal and subterminal endospore were observed under microscopy. Catalase, oxidase activity and nitrate reduction were positive but negative for L-arginine dihydrolase, urease activity and H_2S production. They hydrolyzed casein, gelatin but did not hydrolyze aesculin, starch, Tween 80 and L-tyrosine. They grew in NaCl up to 20% w/v, at pH range 5.0-9.0 and at 10-45°C, but did not grow in 0.5% w/v and at 50°C. They grew under aerobic and anaerobic condition. All produced acids from fructose, galactose,

D-glucose, lactose, maltose, mannitol, mannose, ribose, and sucrose, but did not from L-arabinose, cellobiose, glycerol, inulin, *myo*-inositol, melibiose, melezitose, raffinose, rhamnose, and salicin. Variable on acid production were found in D-xylose, and trehalose.

They contained *meso*-DAP as the diagnostic diamino acid in the cell wall peptidoglycan. The predominant menaquinone was MK-7. The DNA G-C content of CHM 1-4 was 38.0 mol%. On the basis of 16S rDNA sequence and phylogenetic analyses, representative isolate, CHM1-4 in Group I (1,457nt) was clustered within a clade of the genus *Virgibacillus* (Figure 4.2). It was closely related to *V. halodenitrificans* (99.6%) (Table4.6). They showed high DNA-DNA relatedness (74.9-100%) with related isolates (Table 4.3). The high values of DNA-DNA relatedness more than 70% (Wayne et al., 1987), concluded that Group II isolates were identified as *V. halodenitrificans* (Yoon et al., 2004).

Table 4.3 DNA-DNA relatedness of the isolates in Group II and *V. halodenitrificans* JCM 12304^T

Isolate	%DNA-DNA relatedness with labeled strains	
	CHM1-4	**JCM 12304^T**
CHM1-4	100	92.6
BKL1-1	97.5	99.5
BKL2-2	84.1	92.6
BKL6-1	92.7	99.1
CHM1-3	102.2	85.2
AY1-2	95.6	74.9
TP3-1	87.6	95.3
TP4-3	103.9	88.4
TP2-6	99.4	75.7
TSR2-1	ND	88.4
TSY2-3	ND	74.0
BSR3-1	ND	77.0
PT5-1	ND	103.5
V. halodenitrificans JCM 12304^T	94.7	100

ND, not determined.

4.2.3 Group III

Group III contained 13 isolates, BT3-1, BKS1-1, BSR4-1, BY2-4, SB2-2, TM4-3, TPA3-3, ND2-1, PR2-1, PR2-2, RM1-1, TP1-3 and TSR5-2. Cell size was approximate 0.5-0.7 x 2-4 μm. Colonies were circular to slightly irregular, raised, cream in colour (2-6 mm diameter) after 3 days of incubation at 37°C on JCM no. 377 medium. They are motile. Terminal and subterminal endospore were observed under microscopy. Catalase, oxidase, urease activity and nitrate reduction were positive but negative for starch, Tween 80, L-tyrosine and H_2S production. They grew in NaCl between 1-25% w/v, at 15-50°C and at pH range 6.0-8.0. No growth occured in the absence of salts. They grew under aerobic but not anaerobic condition. All produced acid from D-cellobiose, fructose, D-glucose, mannose and salicin but not from L-arabinose, D-galactose, *myo*-inositol, lactose, mannitol, melibiose, melezitose, raffinose, rhamnose trehalose and D-xylose. Variable on acid production were found in glycerol, maltose, ribose and sucrose.

They contained *meso*-DAP in the cell wall peptidoglycan. The predominant menaquinone was MK-7. The DNA G-C content of TP3-3 was 38.8 mol%. On the basis of 16S rDNA sequence and phylogenetic analyses, the representative isolate, TP3-3 (1,545nt) was clustered within a clade of the genus *Virgibacillus* (Figure 4.2). It was closely related to *V. marismortui* (99.6%) (Table 4.6). Isolates MSK2-1, *V. marismortui* KCTC 3867^T and related isolates showed high DNA-DNA relatedness (87.9-100%) (Table 4.4). The high values of DNA-DNA relatedness more than 70% (Wayne et al., 1987), concluded that group III isolates were identified as *V. marismortui* (Arahal et al., 2000).

Table 4.4 DNA-DNA relatedness of the isolates in Group III and *V.marismortui* KCTC 3867^T

Isolate	%DNA-DNA relatedness with labeled strains	
	TP3-3	**KCTC 3867^T**
TP3-3	100	87.9
BY2-4	85.8	100
TP1-3	98.3	89.9
TM4-3	103.2	96.7
SB2-2	93.7	98.3
BT3-1	ND	94.5
PR2-1	ND	71.5
RM1-1	ND	91.9
BKS1-1	ND	90.3
BSR4-1	ND	105.6
PR2-2	ND	89.7
TSR5-2	ND	93.0
ND2-1	ND	92.9
RM2-1	ND	94.1
V. marismortui KCTC 3867^T	98.7	100

ND, not determined.

4.2.4 Group IV

Group IV contained one isolate, MS3-4. Cell size was 0.5-0.7 x 2-5 μm. Colonies were convex, circular with red colour and 2-5 mm in diameter after 3 day of incubation at 37°C on JCM no. 377 medium. Isolate MS3-4 was motile. Terminal and subterminal endospores were observed under microscopy. Catalase, oxidase, hydrolysis of gelatin, starch and L- tyrosine were positive but negative for L-arginine dihydrolase, H_2S production, nitrate reduction, citrate utilization, hydrolysis of aesculin and Tween 80. It hydrolysed casein weakly. This strain grew in NaCl 1-20% w/v, at 15-40°C and at pH range 7.0-8 (optimally in NaCl 5% w/v, at pH 7.0, and at 37°C). MS3-4 grew under aerobic and anaerobic condition. No growth occured in the absence of salts and in 25% w/v. It produced acid from cellobiose, D-glucose, D-maltose, D-ribose, L-sorbose, D-xylose, but did not produce acid from arabinose, D-galactose, glycerol, manose, melezitose, melibiose, ribitol, salicin, sucrose, trehalose and *myo*-inositol.

Strain MS3-4 contained *meso*-DAP as a diagnostic diamino acid in the cell wall peptidoglycan. The predominant quinone was MK-7. The polar lipids analysis revealed the presence of phosphoglycerol (PG), diphosphatidylglycerol (DPG) and an unidentified glycolipid (Figure 4.1).

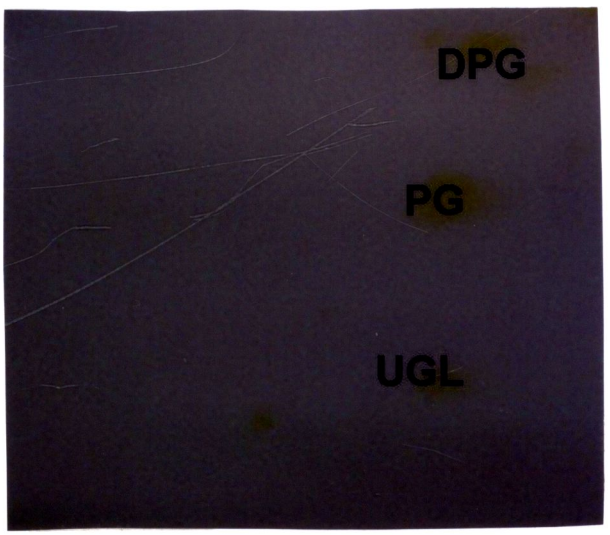

Figure 4.1 Thin-layer chromatogram of the total polar lipids of MS3-4

The cellular fatty acid profile of MS3-4 isolate corresponded to the *Virgibacillus* strains that contained anteiso-$C_{15:0}$ (55.8%), anteiso-$C_{17:0}$ (17.7%), iso-$C_{15:0}$ (11.3%), iso-$C_{16:0}$ (6.61%), iso-$C_{14:0}$ (3.87%), $C_{16:0}$ (1.49%) and iso-$C_{17:0}$ (1.47%) (Table 4.5). The G+C content of DNA of MS3-4 was 38.0 mol%.

On the basis of 16S rDNA sequence and phylogenetic analyses, strain MS3-4 (1,524nt) was closely related to *Virgibacillus* species (93.6-96.0%) and *Lentibacillus* species (93.8-95.4%) (Figure 4.2, Table 4.6). This strain should be the novel species that will be required further studies on the DNA-DNA hybridization with related *Virgibacillus* and *Lentibacillus* species. In this experiment, the type strain *V. carmonensis* KCTC 3819^T was no growth in medium containing KNO_3 under anaerobic condition, in NaCl concentration 0.5- 15% w/v and did not hydrolyse L-tyrosine. *V. carmonensis* KCTC 3819^T produced acid from D-arabinose but not from

D-cellobiose, D-glucose, and D-maltose. The differential characteristics of Group I, II, III, IV and type strains *V. dokdonensis* KCTC 3933^T, *V. halodenitrificans* JCM 12304^T and *V. marismortui* KCTC 3867^{T}were shown in Table. 4.7

Table 4.5 Fatty acid compositions of strain MS3-4, *V. carmonensis* KCTC 3819^T and related species

Strain: 1, MS3-4; 2, *V. carmonensis* KCTC 3819^T (this study); 3, *V. halophilus* 5B73C^T(An et al., 2007); 4, *V. necropolis* (Heyman et al., 2003); 5, *V. dokdonensis* KCTC 3933^T(Yoon et al., 2005); 6, *V. halodenitrificans* JCM 12304^T and 7, *V. marismortui* KCTC 3867^T (Arahal et al., 2000).

Name	Percentage of total[a]						
	1	2	3	4	5	6	7
Saturated straight-chain							
$C_{12:0}$	0.07						
$C_{13:0}$	0.03						
$C_{14:0}$	0.17		2.1				
$C_{15:0}$	0.76		1.4	0.78			39
$C_{16:0}$	1.49	1.03	5.3	1.61		1.06	1.8
$C_{17:0}$	0.08						
$C_{18:0}$							
Saturated branched-chain							
anteiso $C_{11:0}$	0.03						
anteiso $C_{13:0}$	0.21						
iso $C_{14:0}$	3.87	3.32	5.6	2.23		7.44	2.89
anteiso $C_{15:0}$	55.8	58.29	35.8	67.85	34.4	51.8	33.9
iso $C_{15:0}$	11.3	5.73	21.2	3.84	19.4	2.36	
iso $C_{16:0}$	6.61	6.42	10.3	4.16	12.3	11.76	4.33
anteiso $C_{17:0}$	17.7	11.13	16.1	12.19	15.4	19.52	8.55
iso $C_{17:0}$	1.47	0.9	1.8	0.87			5.63
Unsaturated straight-chain							
$C_{16:1}\omega 7c$ alcohol		6.53		2.41		3.05	1.13
$C_{16:1}\omega 11c$		0.63		1.01		0.4	0.64
$C_{18:1}9c$							
SF3[b]		4.98		2.1			
SF4[c]						1.58	0.74

[a]Values are percentage of total cellular fatty acids.

[b]Summed feature 3 contains $C_{16:1}\omega 7c$ and /or iso-$C_{15:0}$ 2OH.

[c]Summed feature 4 contains iso-$C_{17:1}$ and /or anteiso-$C_{17:1}$

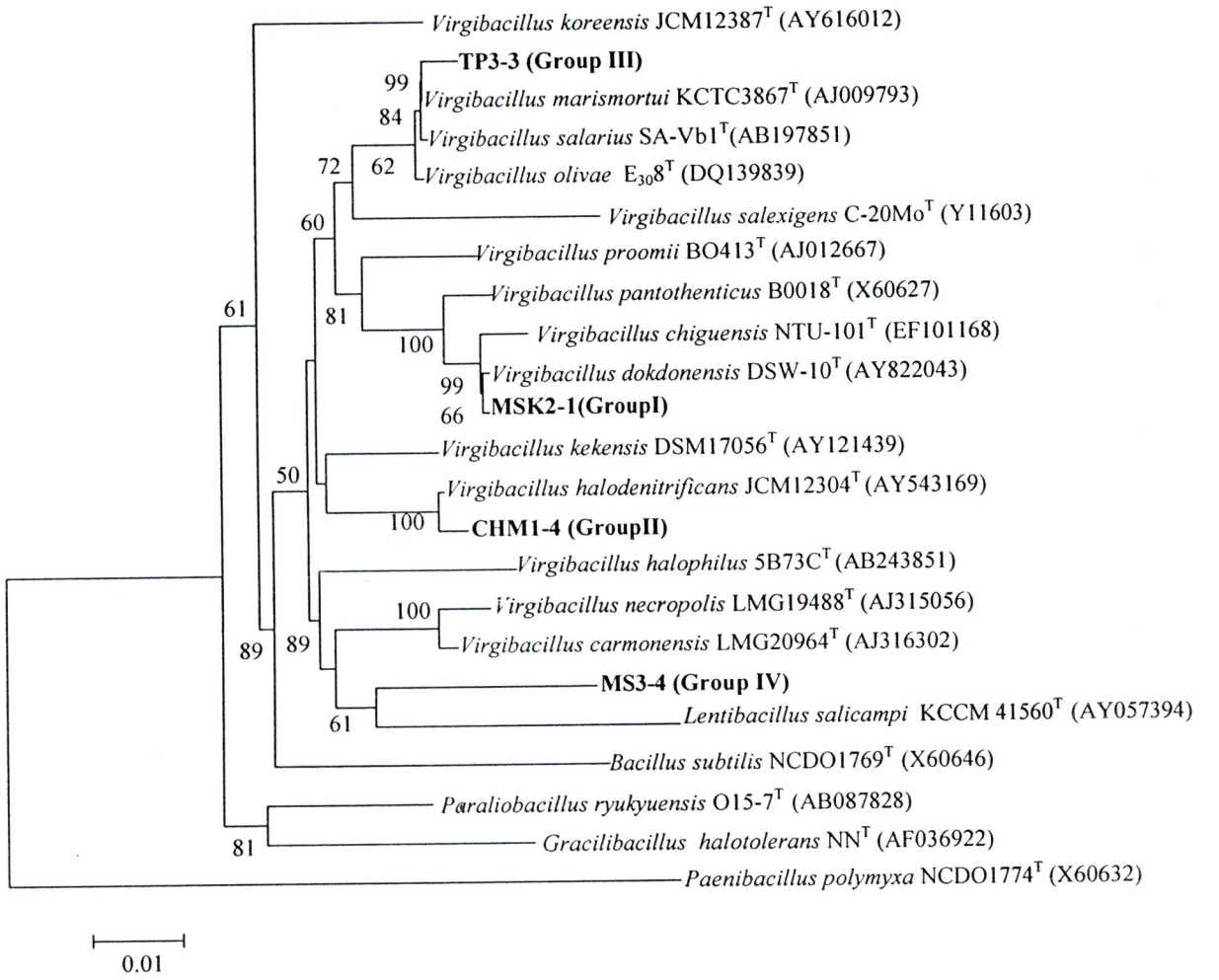

Figure 4.2 Phylogenetic tree showing the relationships between strain MSK2-1, CHM1-4, TP3-3 and MS3-4, *Virgibacillus* species and related taxa based on 16S rDNA sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

Table 4.6 Percentage similarities of MSK2-1 (Group I), CHM1-4 (Group II), TP3-3(Group III), MS3-4 (Group IV) and related taxa.

	Accession no.	% Similarity																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	AB197851	100																						
2	**TP3-3**	99.5	100																					
3	AJ009793	99.9	99.6	100																				
4	DQ139839	99.8	99.4	99.8	100																			
5	Y11603	96.4	96.2	96.5	96.5	100																		
6	EF101168	97	96.6	97.1	97.1	94.8	100																	
7	**MSK2-1**	97.3	97	97.4	97.4	95.2	99.4	100																
8	AY822043	97.3	97	97.4	97.4	95.2	99.4	99.8	100															
9	AJ012667	97.4	97	97.5	97.6	95.3	96.9	97.2	97.2	100														
10	AJ315056	96.2	95.9	96.3	96.1	94.7	95.3	95.8	95.8	96	100													
11	AJ316302	96.6	96.3	96.7	96.5	95	95.5	96	96	96.4	99.2	100												
12	AB087828	94.8	94.5	94.9	94.9	93.1	94.8	95.3	95.3	94.7	94.3	94.9	100											
13	AF036922	93.6	93.3	93.7	93.7	92.4	93.1	93.6	93.6	93.9	93.5	93.8	95.2	100										
14	AY057394	94.7	94.2	94.7	94.7	92.6	93.5	94	94	94.1	94.6	94.1	92.8	92.1	100									
15	**MS3-4**	95.2	94.8	95.3	95.1	93.6	94.2	94.7	94.7	95.2	95.6	96	94.4	93.7	94.1	100								
16	AB243851	96.4	96	96.5	96.3	94.3	94.8	95.3	95.3	95.8	95.6	96.3	94.1	92.8	93.9	94.8	100							
17	AB021186	97.5	97.1	97.6	97.6	95.3	96.3	96.8	96.8	96.3	96.6	97.1	94.8	93.8	94.4	94.8	96.2	100						
18	**CHM1-4**	97.1	96.7	97.1	97.1	95	96.2	96.7	96.7	96	96.5	97	94.5	93.6	94	94.5	95.8	99.6	100					
19	AY616012	96.1	95.8	96.2	96.2	94.5	95.2	95.7	95.7	96	95.6	96.2	95.8	94.4	92.6	93.8	95.2	96.6	96.3	100				
20	AY121439	97.6	97.3	97.7	97.7	95.6	96.1	96.5	96.5	96.8	96.6	97.1	95	94	94.6	95.2	96.4	97.5	97.1	95.8	100			
21	X60627	97.4	97.1	97.5	97.5	95.1	98.5	99	99	97.3	95.7	95.9	94.7	93.4	94.4	94.8	95.3	96.7	96.5	95.7	96.6	100		
22	X60646	94.6	94.2	94.7	94.7	92.7	93.9	94.4	94.4	94	93.6	93.9	93.4	92.5	91.4	92.8	93.5	94.5	94.4	94	94.1	93.8	100	
23	X60632	87	86.6	87.1	87.1	85	86.6	87.1	87.1	87.8	87.3	87.8	87.9	86.4	86.1	86.8	87.5	86.5	86.3	87.5	87.8	86.9	86.1	100

Table 4.7 Differential characteristics of MS3-4 and isolates in Group I, II, III and IV and *V. dokdonensis* KCTC 3933^T, *V. halodenitrificans* JCM 12304^T and *V. marismortui* KCTC 3867^T and *V. carmonensis* KCTC 3819^T. (Data in this study)

Characteristics	Group I (10)	Group II (9)	Group III (5)	Group IV (1)	KCTC 3933^T	JCM 12304^T	KCTC 3867^T	KCTC 3819^T
Colony colour	Milky white	Creamy brown	Creamy white	red	Milky white	Creamy brown	Creamy white	Pink tint
Spore shape	E,S	S E	E	E	E,S	E	E	E,S
Spore position	T,ST	T,ST	T,ST	ST	T,ST	T,ST	T,ST	ST
Anaerobic growth	+	+	-	+	+	+	-	-
Growth at/in:								
50° C	+(-3)	-	+	-	+	-	+	-
0 % NaCl	+	-	-	-	+	-	-	-
20% NaCl	W	W	+(-2)	+	W	+	+	-
pH range	5-8	5-9	6-8	7-8	5-8	5-9	6-8.5	ND
Hydrolysis of:								
Aesculin	+	-	+	-	+	-	+	W
Casein	+	+	+	W	+	+	+	+
Gelatin	+	+	+	+	+	+	+	-
Starch	+	-	-	+	+	-	-	ND
Tween 80	+(-2)	-(+1)	-	-	+	-	-	
Nitrate reduction	-	+	+	+	-	+	+	+
Acid from:								
L-Arabinose	+	-	-	-	+	-	-	NG
Cellobiose	+	-	+	+	+	-	+	NG
D-Fructose	+	+	+	-	+	+	+	-
D-Galactose	+	+	-	-	+	+	-	-
D-Glucose	+	+	+	+	+	+	+	-
Glycerol	+	-	+(-1)	-	+	-	+	NG
myo-Inositol	+	-	-	-	+	-	-	-
Inulin	+	-	ND	ND	+	-	ND	
Lactose	+	+	-	-	+	+	-	NG
Mannitol	+	+	-	ND	+	+	-	NG
D-mannose	+	+	+	-	+	+	+	-
Melezitose	+	-	-	-	+	-	-	NG
Salicin	+	-	+	-	+	-	+	NG
Sucrose	+	+	-(+1)	-	+	+	-	NG
D-Xylose	+	-(+2)	-	+	+	-	-	NG
Major polar lipid	DPG, PG, PE,PLs	DPG, PG, PLs	DPG, PG, PE,PLs	DPG, PG	DPG, PG, PE,PLs	DPG, PG, PLs	DPG, PG, PE,PLs	DPG, PG
DNA G+C (mol%)	36.5	38.0	38.8	38.0	36.7	38.0	39.0-42.8	38.9

+, positive; - negative; ND, no data; NG, no growth

4.2.5 Group V

Group V contained only one isolate, TP2-8. Cell size was approximate 0.2-0.4 μm wide by 0.3-0.6 μm long. Colonies were irregular, translucent, and white colour (2-5 mm diameter) after 3 days of incubation at 37°C on JCM no. 377 medium. Motile by means of peritrichous flagella were observed under microscopy. Oval endospores were produced at a terminal position in swollen sporangia (Figure 4.3).

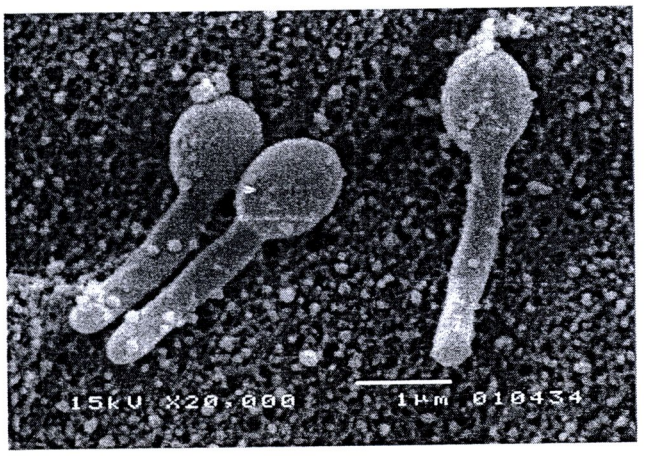

Figure 4.3 Scanning electron micrograph of TP2-8 on JCM no. 377 medium at 37°C for 3 days

The isolate grew at pH range 5.0-8.0 (optimally at pH 7.0), at 14-55°C (optimally at 37 °C), and in 1 to 22 % w/v NaCl (optimally in 5-10 % w/v) but not in 0.5 % w/v or below. This strain was no growth under anaerobic conditions even KNO_3 was added. Oxidase and catalase, hydrolysis of gelatin, esculin, nitrate reduction were positive, but casein, starch, Tween 80, L-tyrosine, arginine dihydrolase and H_2S were negative. Sensitive to ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), novobiocin (30 μg) and rifampicin (30 μg), but resistant to bacitracin (10 units), chloramphenicol (30 μg), nalidixic acid (30 μg). Acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, melibiose, melezitose, raffinose, rhamnose, ribose, salicin, sucrose, trehalose and D-xylose but not from inulin, *myo-*

inositol, lactose, sorbitol, ribitol and arabitol. Utilized sodium citrate, lactose, D-mannose, D-raffinose, sodium benzoate, melezitose, trehalose, galactose, L-arginine, L-aspartic acid, L- ornithine, but not sodium acetate, sodium pyruvate, hippuric acid, L-lysinemonohydrochloride, L-glutamic acid, L-phenylalanine, L-cysteine.

The strain TP2-8 contained MK-7 as the major menaquinone. Major cellular fatty acids are anteiso-$C_{15:0}$ (37.6%), iso-$C_{15:0}$ (16.1%), and anteiso-$C_{17:0}$ (12.9%). The cellular fatty acid profile of TP2-8 isolate corresponded to *Gracilibacillus* specie as shown in Table 4.8. It contained diphosphatidyl glycerol, phosphatidylglycerol, and unidentified glycolipid as polar lipids (Figure 4.4). The DNA G+C content was 37.6 mol%.

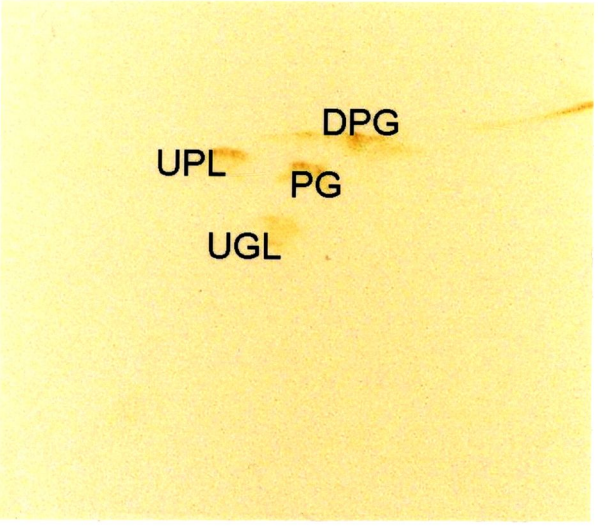

Figure 4.4 Thin-layer chromatogram of polar lipids of TP2-8

Table 4.8 Cellular fatty acid composition of strain TP2-8 and related *Gracilibacillus* species

Strains: 1, TP2-8; 2, *G. saliphilus* YIM 91119^T; 3, *G. lacisalsi* DSM 19029^T; 4, *G. orientalis* CCM 7326^T; 5, *G. quinghaiensis* DSM 17858^T; 6, *G. boraciitolerans* JCM 7214^T; 7, *G. dipsosauri* JCM 7303^T. Data were taken from this study and from Ahmed et al. (2007), Carrasco et al. (2006), Jeon et al. (2008), Chen et al. (2008a, 2008b).

Fatty acid	Percentage of total[a]						
	1	2	3	4	5	6	7
Saturated straight-chain							
$C_{14:0}$	1.0	0.8	1.4		3.7	0.7	
$C_{15:0}$	5.0	3.6	10.9		1.4	1.9	
$C_{16:0}$	7.9	7.2	9.8	4.9	17.4	5.3	7.2
$C_{18:0}$	1.0	1.0	1.0		4.1	0.7	
$C_{17:0}$	3.8	3.0	4.4	3.2		0.5	
Unsaturated straight-chain							
$C_{16:1}\omega 7c$					2		
$C_{16:1}\omega 11c$	2.8	0.5	ND		10.8	0.2	
$C_{18:1}\omega 7c$					1.4		
$C_{18:1}\omega 9c$		0.9			1.3	1	
$C_{16:1}\omega 7c$ alcohol	1.2						
Saturated branched-chain							
iso-$C_{13:0}$			0.5				
iso-$C_{14:0}$	2.8	0.5	1.1	1	0.7	0.6	
iso-$C_{15:0}$	16.1	22.2	15.1	8.5	11.5	18.2	17.6
anteiso-$C_{15:0}$	37.6	32.9	36.7	51.9	28.3	45.7	39.1
iso-$C_{16:0}$	4.8	1.8	2.4	3.3	1	1.9	4.7
anteiso-$C_{16:0}$		0.6					
iso-$C_{17:0}$	2.5	6.1		2.4	1.8	3.2	7.3
anteiso-$C_{17:0}$	12.9	16.5	12.3	20.6	10.8	16.9	24.2
anteiso-$C_{19:0}$	0.15	0.7				0.8	
Hydroxylated fatty acids							
iso-$C_{13:0}$ 3OH					1.25		
SF 5[b]		0.7					

+, Positive; w, weakly positive; -, negative; A, aerobe; FA, facultative anaerobe; ND, no data.

[a]Values are percentage of total cellular fatty acids.

[b]Summed feature 5 contained $C_{18:2}\omega 6,9c$ and /or anteiso-$C_{18:0}$

Almost complete 16S rDNA sequence of TP2-8 (1,551nt) was analyzed. A tree constructed by the neighbour-joining algorithm analysis clearly showed that the TP2-8 was placed in the genus *Gracilibacillus* (Figure 4.5). Topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony analysis were similar to those of the tree constructed by maximum-parsimony analysis (Appendix F). The comparative 16S rDNA sequence analyses showed that strain TP2-8 was closely related *to G. saliphilus* YIM 91119^T (99.2%), *G. lacisalsi* DSM 19029^T (98.6%), *G. orientalis* CCM 7326^T (97.7%), *G. quinghaiensis* DSM 17858^T (97.7%), *G. boraciitolerans* JCM 7214^T (97.2 %), *G. dipsosauri* JCM 7303^T (96.4%), *G. halotolerans* JCM 7302^T (95.8 %), and *G. halophilus* DSM 17856^T (94.9%) (Table 4.9).

DNA-DNA hybridization studies, showed that strain TP2-8 had low levels of DNA-DNA relatedness to *G. saliphilus* YIM 91119^T (45%), *G. lacisalsi* DSM 19029^T (32.0%), *G. orientalis* CCM 7326^T (34%), G. *quinghaiensis* DSM 17858^T (17%), *G. boraciitolerans* JCM 21714^T (21%), *G. halotolerans* JCM 7303^T (19%), *G. dipsosauri* JCM 7302^T (6%), and *G. halophilus* DSM 17856^T (12%). In addition, isolate TP2-8 was differentiated from *Gracilibacillus* species by some physiological and biochemical properties (Table 4.10). This strain should be the novel halophilic bacterium and was named as *Gracilibacillu thailandensis*. The type strain is TP2-8 (JCM 15569^T =PCU 304^T =TISTR 1881^T).

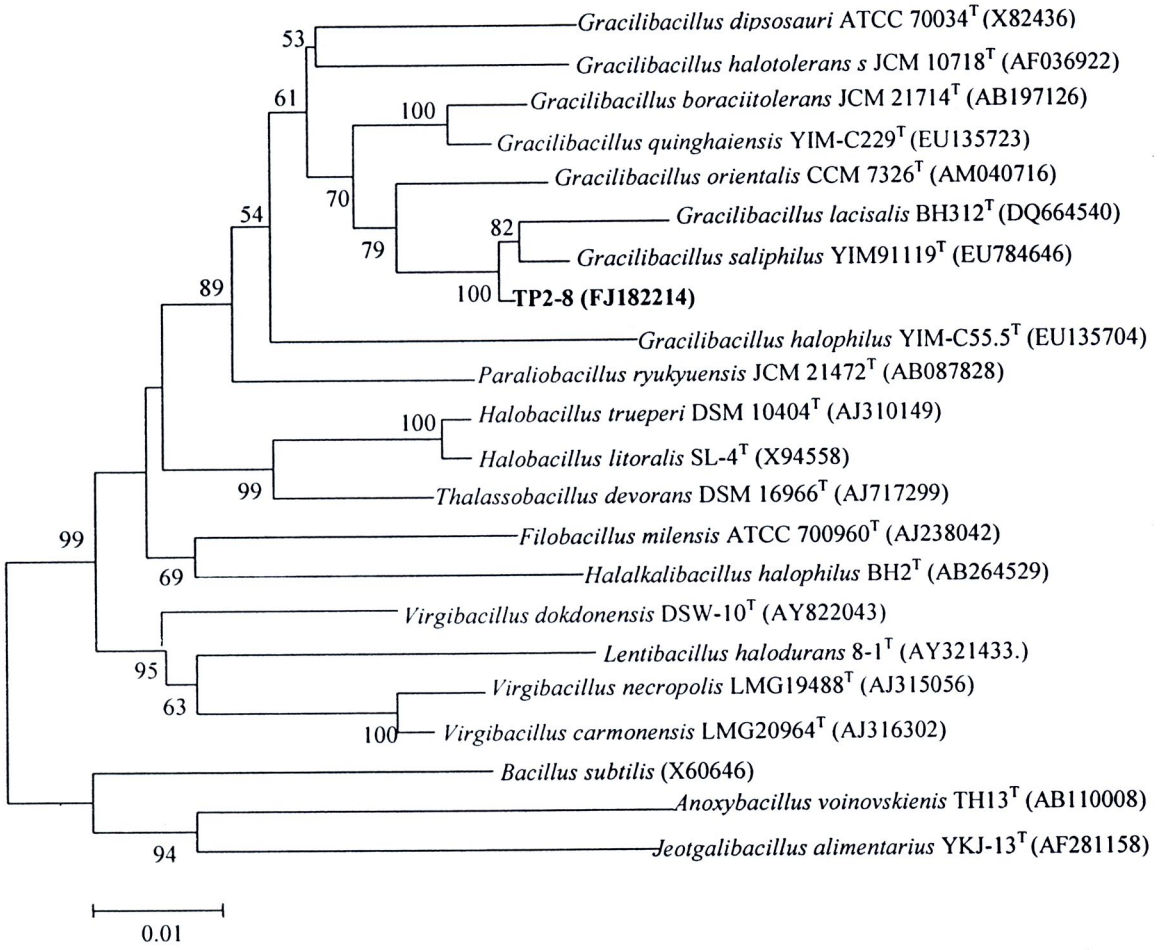

Figure 4.5 Phylogenetic tree showing the relationships between strain TP2-8^T, *Gracilibacillus* species and related taxa based on 16S rDNA sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position

Table 4.9 Percentage similarities of Group V (TP2-8) and related taxa.

	Accession no.	% Similarity																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	AJ315056	100																					
2	AJ316302	99.1	100																				
3	AY321433	94.2	94.9	100																			
4	AY822043	95.3	95.6	94.1	100																		
5	X94558	94.2	94.6	92.9	94.3	100																	
6	AJ310149	94.2	94.6	92.5	94.3	99.5	100																
7	AJ717299	93.8	94.3	93	94.7	96.9	97	100															
8	DQ664540	92	92.4	91.3	93.1	93.6	93.4	93.6	100														
9	EU784646	92.8	93.3	92	93.8	94.5	94.4	94.4	98.5	100													
10	**TP2-8**	93.1	93.6	92.5	94.2	94.9	94.7	94.7	98.6	99.3	100												
11	AM040716	93.4	93.5	92.7	93.3	94.9	94.7	94.6	96.3	97.4	97.72	100											
12	EU135723	93.5	94	92.9	94.3	95.2	95.2	95.9	96.7	97.1	97.71	97	100										
13	AB197126	93	93.4	92.4	93.9	94.8	94.8	95.5	96.2	96.7	97.26	96.8	99.1	100									
14	AF036922	93.4	93.6	92.6	93.6	94.1	94.4	93.6	94.6	95.3	95.81	95.5	96.2	95.7	100								
15	X82436	92.5	92.8	91.9	92.7	93.6	93.7	93.7	95	96.1	96.41	96	95.7	95.7	95.6	100							
16	EU135704	92.4	92.8	92.7	93.1	93.6	93.9	93.4	93.5	94.5	94.93	95.3	94.6	94.5	94.2	94.3	100						
17	AB087828	94.1	94.6	93	95.1	94.6	94.6	94.3	94.6	95.4	95.88	95.1	95.7	96	95.3	95.2	94.7	100					
18	AB264529	92.2	92.7	92.1	92.1	92.4	92.4	94.2	92.3	93.2	93.58	93.1	93.8	93.6	92.8	93.3	91.9	92.9	100				
19	AJ238042	92.9	93.5	92.3	93.6	94.3	94.1	94.1	93.2	94.1	94.46	93.8	94.1	94.1	93.6	93.7	93.1	93.8	93.9	100			
20	X60646	92.5	92.7	90.9	92.9	92.2	92.1	92	90.9	91.5	91.96	91.5	91.6	91.6	91.8	92.2	90.7	92.6	91.7	92.5	100		
21	AB110008	91	91.2	90.1	91.5	91.3	91.6	92.3	88.7	89.2	89.84	89.7	90.4	90.1	90.2	90	89.5	90.9	90.7	91.3	91.7	100	
22	AF281158	90.7	91.1	89.4	91.4	90.4	90.4	91.2	88.6	89.1	89.68	89	89.8	89.9	89.7	89.3	88.4	91.1	90.8	90.8	92.1	92.4	100

Table 4.10 Differential characteristics of Group V, TP2-8 and related *Gracilibacillus* species

Strains: 1, TP2-8; 2, *G. saliphilus* YIM 91119^T; 3, *G. lacisalsi* DSM 19029^T; 4, *G. orientalis* CCM 7326^T; 5, *G. quinghaiensis* DSM 17858^T; 6, *G. boraciitolerans* JCM 7214^T; 7, *G. dipsosauri* JCM 7303^T. Data were taken from this study and from Ahmed et al. (2007), Carrasco et al. (2006), Jeon et al. (2008), Chen et al. (2008a, 2008b).

Characteristic	1	2	3	4	5	6	7
Colony colour	W	CW	C	C	CW	P	W
Spore shape	O	S	S	S	E	S	S
Oxygen requirement	A	A	A	A	A	ND	FA
NaCl range (%, w/v)	1-22	1-22	0.5-18	3-20	0.5-8	0-11	0-15
Optimum growth in NaCl	5-10	10-15	5-7	10	1-3	0.5-3	3
Temperature range (°C)	14-55	4-45	15-50	4-45	4-45	11-37	28-50
Optimum temperature	37	28-37	37	37	37	25-28	45
pH range	6-8	6-8	5.5-10	5-9	6-8.5	6-10	ND28-
Optimum pH	7		7.5-8	7.5	7-7.5	7.5-8.5	7.5
Nitrate reduction	+	+	+	-	+	-	+
Hydrolysis of:							
Gelatin	+	-	-	+	-	-	+
Starch	-	+	-	-	-	+	-
Acid from:							
Glycerol	+	-	+	-	w	+	-
Lactose	-	+	-	-	-	+	+
Melezitose	+	-	-	-	-	+	-
G+C content (mol%)	37.6	40.1	39	37.1	40.9	35.8	39.4

+, Positive; w, weakly positive; -, negative; A, aerobe; FA, facultative anaerobe; C, cream; CW, cream white; LP, light pink; W, white; E, ellipsoidal; O, oval; S, spherical; ND, no data.

4.2.6 Group VI

Group VI contained six isolates, TPA3-2, HR1-1, MS2-6, AG1-3, AG2-2 and BY1-1. Colonies were circular to slightly irregular in shape, yellow pigment (1- 3 mm diameter) after 3 days of incubation at 37°C on JCM no. 377 medium. They are motile. Subterminal endospores were observed under microscopy. Catalase and oxidase were positive but negative for urease activity and L-arginine dihydrolase, nitrate reduction and H_2S production. They hydrolyzed aesculin, casein, and gelatin but not hydrolyzed L-tyrosine. Hydrolysis of starch and Tween80 were variable. They grew in NaCl concentration between 5-20% w/v, but not grew in absence of NaCl. They grew in pH range 7.0-8.0 and at 20-45°C. Optimum for growth were 10% NaCl, pH 7-8, at 37°C. They grew under aerobic but not anaerobic condition. Acid was from D-fructose, D-glucose, D-ribose and sucrose but not produced from L-arabinose, D-cellobiose, D-galactose, D-mannose, melibiose, maltose, maltose, D-mannitol, D-raffinose, D-trehalose and D-xylose.

The major menaquinone was MK-7. The cell wall peptidoglycan was the L-Orn type. The major fatty acids were saturated branched-chain (Table 4.11). The G+C content of TPA3-2 was 41.0 mol % . On the basis of 16S rDNA sequence and phylogenetic analyses, the representative isolate, TPA3-2 in Group VI (1,521nt) was clustered within a clade of the genus *Halobacillus* (Figure 4.6). Strain TPA3-2 showed sequence similarity of 99.5-97.0% to the type strain of *Halobacillus* species. On the other hand, 16S DNA sequence similarities of less than 97.0% were obtained with other related genera (Table 4.12). The phylogenetic tree indicated that strain 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%). From the result of DNA-DNA relatedness (Table 4.13) isolate TPA3-2 showed low level DNA-DNA relatedness less than 70% with *H. locisalis* KCTC 3788^T and BY1-1. However, TPA3-2 showed high DNA-DNA relatedness with AG1-3, AG2-2 and HR1-1 but showed lower than 70% with isolates BY1-1 and MS2-6. This indicated that Group VI isolates were contained two species that be required further studies on the 16S rDNA sequencing and DNA-DNA hybridization. The differential characteristics of isolates and relates *Halobacillus* species were shown in Table 4.14.

Table 4.11 Cellular fatty acid composition of strain TPA 3-2, MS2-6 and related *Halobacillus* species.

Strains: 1, TPA3-2; 2 MS2-6; 3, *H. locisalis* MSS-155 ^{T}; 4, *H. trueperi* DSM 10404^{T}; 5, *H. dabanensis* D-8^{T}; 6, *H. faecis* IGA7-4^{T}.

Fatty acid	Percentage of total[a]					
	1	2	3	4	5	6
Saturated straight-chain						
$C_{15:0}$		0.8	1.1		2.9	1.2
$C_{16:0}$	0.9	1.0	0.9	0.9	2.0	2.1
Unsaturated straight-chain						
$C_{16:1}\,\omega 11c$	0.5	0.3			0.7	
$C_{16:1}\,\omega 7c$ alcohol	6.9	1.3	5.7	3.8	4.1	1.9
$C_{17:1}\,\omega 10c$					0.4	
Saturated branched-chain						
anteiso-$C_{13:0}$	0.6	0.8			0.1	
iso-$C_{14:0}$	13.0	2.4	11.2	21.7	1.1	2.5
iso-$C_{15:0}$	12.0	9.7	8.4	7.7	7.5	13.8
anteiso-$C_{15:0}$	30.5	62.1	42.0	25.3	49.7	50.0
iso-$C_{16:0}$	17.1	4.4	15.9	31.5	8.2	9.8
iso-$C_{17:0}$	2.0	1.3	1.4	2.1	2.2	2.9
anteiso-$C_{17:0}$	8.7	12.6	13.0	6.5	18.6	15.0
Unsaturated branched-chain						
anteiso-$C_{13:1}$			0.5			
Hydroxylated fatty acids						
$C_{16:1}\,\omega 7c$ alcohol	6.91	1.3	5.7	3.8	4.1	1.9
SF3[b]						
SF4[c]	1.06	2.7				

[a]Values are percentage of total cellular fatty acids.

[b]Summed feature 3 contains $C_{16:1}\omega 7c$ and /or iso-$C_{15:0}$ 2OH

[c]Summed feature 4 contains iso-$C_{17:1}$ and /or anteiso $C_{17:1}$

.

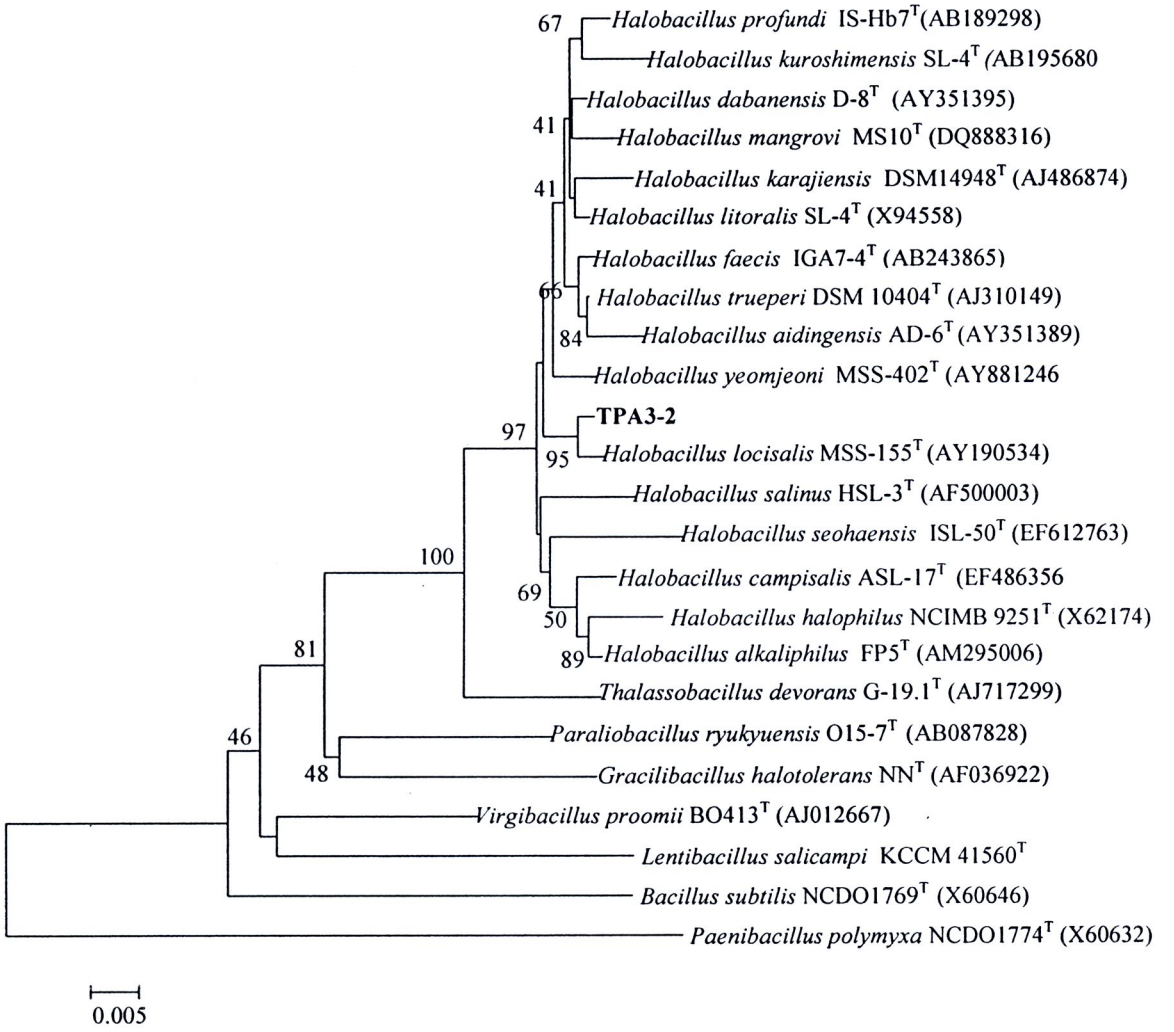

Figure 4.6 Phylogenetic tree showing the relationships between strain TPA3-2, *Halobacillus* species and related taxa based on 16S rDNA sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.005 substitutions per nucleotide position

Table 4.12 Percentage similarities of TPA3-2 (Group VI) and related taxa.

	Accession no.	% Similarity																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	AJ012667	100																							
2	AY057394	94.2	100																						
3	AB195680	93.7	91.9	100																					
4	10X62174	93.7	91.8	97.5	100																				
5	DQ888316	94.1	92.9	98.5	97.8	100																			
6	**TPA3-2**	94.4	92.6	98.3	97.8	98.5	100																		
7	AY190534	94.1	92.5	98.2	97.7	98.5	99.5	100																	
8	AM295006	94.1	92.3	98.2	99.1	98.6	98.5	98.4	100																
9	EF486356	93.9	92.2	98.2	98.6	98.3	98.5	98.4	99.4	100															
10	EF612763	93.8	92.1	97.4	97.4	97.3	97.6	97.6	97.7	98.3	100														
11	AF500003	93.8	92	98	97.6	97.8	98.5	98.5	98.4	98.4	97.5	100													
12	AY881246	94.2	92.7	98.2	98	98.7	98.9	98.9	98.7	98.4	97.6	98.5	100												
13	AJ310149	94.2	92.4	98.9	98.1	99	99	98.9	98.8	98.6	97.9	98.4	99.2	100											
14	AY351389	93.5	91.7	98.4	97.5	98.4	98.5	98.5	98.2	98.1	97.4	97.8	98.8	99.4	100										
15	AB243865	94.1	92.3	98.8	98.3	98.7	99	99	98.8	98.6	97.8	98.4	99.2	99.7	99.2	100									
16	X94558	94.3	92.6	98.9	98.2	99.1	98.9	98.9	99	98.7	97.9	98.6	99.2	99.4	98.9	99.3	100								
17	AJ486874	93.7	91.9	98.5	97.7	98.7	98.3	98.3	98.4	98.1	97.3	97.8	98.8	99.2	98.8	99	99.2	100							
18	AB189298	94	92.2	99	98.1	99.1	98.7	98.5	98.8	98.5	97.5	98.2	98.8	99.3	98.8	99.2	99.5	99.1	100						
19	AY351395	94.3	92.5	98.9	98.2	99.3	99	98.9	98.9	98.8	97.7	98.2	99.2	99.5	99	99.4	99.5	99.3	99.5	100					
20	AJ717299	94	92.7	96.6	96.6	97.3	97	96.6	97.4	97.1	95.7	96.6	97.3	97.1	96.6	97	97	96.8	96.9	97.4	100				
21	AB087828	94.4	93	94.2	94.1	94.4	95	94.9	94.5	94.4	94	94.5	94.7	94.9	94.2	94.9	94.8	94.4	94.5	94.9	94.5	100			
22	AF036922	93.7	92.4	93.9	93.6	94.2	94.5	94.2	94.2	94.1	94.1	94	94.3	94.6	94	94.5	94.2	94	94.2	94.6	93.9	95.1	100		
23	X60646	92.6	91.1	91.4	90.9	91.9	92.3	92.2	91.7	91.5	91	91.8	92	92.1	91.4	92.2	92.1	91.6	91.7	92.1	92.1	92.6	91.5	100	
24	X60632	88.2	86.9	85.9	86.2	86.5	86.7	86.5	86.3	86.1	86.2	85.8	86.3	86.6	85.8	86.5	86.5	86.1	86.3	86.6	86.9	88.2	86.6	86.3	100

Table 4.13 DNA-DNA relatedness of the isolates in Group VI and *H. locisalis* KCTC 3788^T

Isolates	% of DNA-DNA relatedness with label strains	
	TPA3-2	KCTC 3788^T
TPA3-2	100	18.0
AG1-3	95.3	25.4
AG2-2	99.5	27.6
HR1-1	98.6	28.6
BY1-1	20.7	39.9
MS2-6	38.02	27.6
H. locisalis KCTC 3788^T	38.02	100

Table 4.14 Differential characteristics between Group VI and *Halobacillus* species

Strains: 1, Group VI; 2, *H. locisalis* MSS-155^T; 3, *H. trueperi* DSM 10404^T; 4, *H. dabanensis* D-8^T ; 5, *H. faecis* IGA7-4^T. Data were taken from this study and from Amoozegar et al., 2003; Spring et al., 1996.

Characteristics	Group VI (6 isolates)	1	2	3	4
Spore shape	E	E	S	S	S
Spore position	C,ST	C,ST	C,ST	C,ST	C
Pigmentation	LOY	LOY	Orange	Orange	Orange
NaCl (%) range	10	1-23	0.5-30	0.5-25	0-15
NaCl (%) optimum	5-20	10	10	10	ND
Temp. range (°C)	20-45	10-42	10-44	15-50	15-45
pH range	5-9	5-9.5	6-9.5	5-11	5.5-9
Hydrolysis of: Aesculin	+	+	-	-	ND
Casein	+	-	-	+	ND
Gelatin	+	-	+	-	+
Starch	+(-1)	+	-	+	ND
Tween 80	-(+1)	w	-	-	+
Acid from: D-Cellobiose	-	+	ND	ND	ND
Galactose	-	-	+	-	+
Trehalose	-	+	+	+	+
D-Xylose	-	-	-	+	-
G+C content (mol%)	41	44	43	41.4	46.5

LOY, light orange yellow; O, orange. +, Positive; w, weakly positive; -, negative; A, aerobe; FA, facultative anaerobe; ND, no data. +, positive; - negative; ND, no data.

4.2.7 Group VII

Group VII comprised ten isolates, N20-1, TPA1-1, MS1-3, MS3-2, NS1-7, PJ1-6, TPA1-7, TPA4-4, TPA4-6 and TPA4-7. Colonies were irregular, raise, pale orange yellow in colour (0.5 – 3.5 mm diameter) after 3 days of incubation at 37°C on JCM no. 377 medium. They are motile. Central and subterminal endospore were observed under microscopy. They grew in NaCl concentration between 0-15 % w/v but optimal growth in 0 - 1% NaCl. Growth in pH range 5.0-8.0, at temperature range 20-45°C. They grew under aerobic and anaerobic condition. Catalase and oxidase activity were positive but negative for H_2S formation. They hydrolyzed casein, starch, DNA, aesculin, and gelatin but did not hydrolyze L-tyrosine. Acid was produced from D-fructose, D-glucose, raffinose, salicin, sorbitol but no acid is produced from D-cellobiose, galactose, *myo*-inositol, melibiose, sucrose, and trehalose. Variable acid production was found in D-mannose.

Major quinone was MK-7. *meso*-DAP was found in the cell walls. DNA G+C content of N20-1 was 38.0 mol%. On the basis of 16S rDNA sequence and phylogenetic analyses, the representative isolate, N20-1 in Group VII (1,148 nt) was a member of the genus *Bacillus* (Figure 4.7). The comparative 16S rDNA sequence analyses showed that strain N20-1 was closely related to *B. vietnamensis* (99.6%), *B. aquimaris* (99.3%) and *B. marisflavi* (98.4%) (Table 4.15).

Isolate N20-1 and TPA4-7 showed high DNA-DNA relatedness to each other but showed low DNA-DNA relatedness with *B. marisflavi* KCTC 3906^T and *B. aquimaris* KCTC3903^T. The remained 8 isolates also showed high DNA-DNA relatedness (98.6-100.4%) to each other as the same species (Table 4.16). The further studies on the 16S rDNA sequencing and DNA-DNA hybridization are required. The differential characteristics of Group VII isolates and *B. vietnamnensis* JCM 11124^T, *B. marisflavi* KCTC 3906^T and *B. aquimaris* KCTC 3903^T were summarized in Table 4. 17.

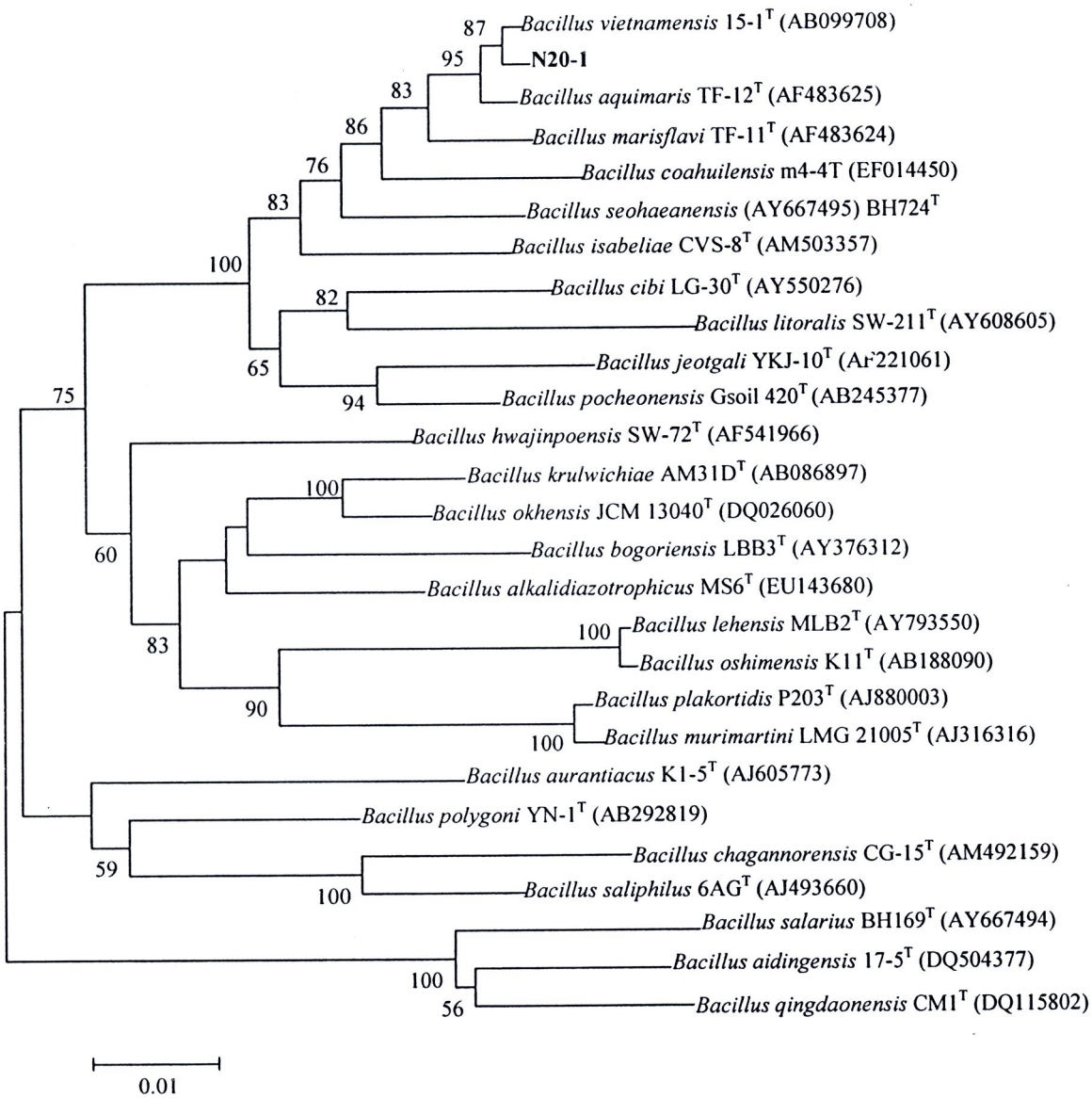

Figure 4.7 Phylogenetic tree showing the relationships between strain N20-1, *Bacillus* species and related taxa based on 16S rDNA sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position

Table 4.15. Percentage similarities of N20-1 (Group VII) and related taxa.

	Accession no.	% Similarity																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	EF014450	100																						
2	AM503357	95.4	100																					
3	AY550276	94.3	95.8	100																				
4	AY608605	94.3	94.5	95.5	100																			
5	AF221061	94.6	94.5	95.5	94.5	100																		
6	AB245377	95.8	95.9	95.4	95.0	97.3	100																	
7	AB099708	97.5	96.4	94.8	94.6	95.0	96.1	100																
8	**N20-1**	97.2	96.5	94.9	94.5	94.7	96.0	99.6	100															
9	AF483625	97.0	96.6	94.8	95.0	94.8	96.3	99.2	99.3	100														
10	AF483624	96.9	96.4	95.6	94.9	94.5	96.0	98.4	98.4	98.4	100													
11	AY667495	96.3	96.8	95.5	94.4	93.7	95.7	97.2	97.2	97.6	97.1	100												
12	AF541966	93.3	94.1	93.1	92.2	93.2	94.7	94.0	94.0	94.5	94.1	94.0	100											
13	AB086897	92.6	93.2	92.6	91.2	91.9	93.3	92.5	92.5	92.7	92.9	93.6	95.2	100										
14	DQ026060	92.8	93.8	93.0	91.0	92.6	93.4	92.9	92.8	93.3	92.8	93.7	95.3	98.2	100									
15	AY376312	93.1	93.0	92.5	91.3	92.4	93.1	93.3	93.2	93.2	92.7	93.2	94.2	95.8	96.4	100								
16	EU143680	93.2	93.7	93.3	92.3	93.2	94.2	93.1	93.0	93.3	92.9	93.6	95.5	96.6	96.8	95.9	100							
17	AY793550	91.8	91.7	92.6	91.4	92.6	92.5	91.6	91.5	91.8	91.1	91.7	93.2	93.1	94.1	94.4	94.4	100						
18	AB188090	91.8	91.6	92.6	91.2	92.4	92.5	91.6	91.5	91.8	91.1	91.7	93.2	93.2	94.1	94.3	94.3	99.8	100					
19	AJ880003	91.7	93.1	92.3	90.5	91.5	92.2	91.8	91.8	91.9	92.3	92.6	93.8	94.4	94.8	93.5	94.4	94.7	94.6	100				
20	AJ316316	91.6	92.9	92.1	90.3	91.5	92.0	91.8	91.8	91.9	92.3	92.8	93.7	94.3	94.6	93.5	94.2	94.5	94.4	99.6	100			
21	AJ605773	92.5	92.5	92.6	91.7	91.5	92.5	93.8	93.8	94.0	93.7	92.5	93.5	92.6	92.7	92.8	93.7	91.3	91.2	92.0	91.8	100		
22	DQ115802	90.3	90.5	90.3	89.0	90.9	90.3	90.1	90.0	90.2	90.3	89.8	90.1	90.2	90.5	88.9	90.1	88.6	88.6	90.7	90.6	90.0	100	
23	AY667494	90.1	90.2	90.7	88.6	91.0	90.0	89.9	89.7	89.8	89.7	90.5	90.8	90.9	91.3	89.6	90.4	89.5	89.4	90.4	90.5	89.5	95.8	100

Table 4.16 DNA-DNA relatedness of the isolates in Group VII

Isolate	% of DNA-DNA relatedness	
	N20-1	TP4-6
N20-1	100	39.5
TPA4-7	102.1	48.3
TPA1-1	27.5	100
MS1-3	40.9	99.5
MS3-2	29.3	98.6
NS1-7	42.2	100.2
TPA4-4	50.6	99.1
TPA4-6	48.1	100
TPA1-7	36.5	98.9
PJ1-6	14.3	100.4
B. aquimaris KCTC 3903^T	20.7	13.9
B. mariflavi KCTC 3906^T	23.6	25.6

Table 4.17 Differential characteristics of Group VII isolates and *B. vietnamensis* JCM 11124^T, *B. marisflavi* KCTC 3906^T and *B. aquimaris* KCTC3903^T

Characteristics	**Group VII (10 isolates)**	**JCM 11124^T**	**KCTC 3906^T**	**KCTC 3903^T**
Pigmentation	Cream orange yellow	Cream orange yellow	Pale yellow	Pale orange-yellow
Temperature range (°C)	10-45	10-45	10-47	10-44
NaCl range (%)	0-15	0-15	0-16	0-18
pH range	5-8	5-10	4.5-7	4.5-9
Oxidase	+	+	-	-
Hydrolysis of				
Esculin	+	+	+	-
Gelatin	+	+	ND	ND
Starch	+	+	-	+
Tyrosine	+	+	-	-
Acid production from				
D-Cellobiose	-	-	+	+
Galactose	-	-	w	+
myo-Inositol	-	+	-	+
Mannose	+(-2)	-	+	+
Melibiose	-	-	+	+
Sucrose	-	-	+	+
Sorbitol	+	+	-	-
Trehalose	-	+	+	+
G+C content (mol%)	38	43	49	38

+, positive; - negative; ND, no data.

4.2.8 Group VIII

Strain ND1-1 was Gram-negative, non-endospore-forming, curved rods, approximately 0.4-0.5μm wide by 2-8 μm long. Colonies were round with entire edges, smooth, convex, opaque and cream pigmented, diameter reached 0.9-2.5 mm after 3 days of incubation at 37 °C on JCM medium no. 377 agar (Figure 4.8A). Motile by means of one polar flagellum (Figure 4.8B).

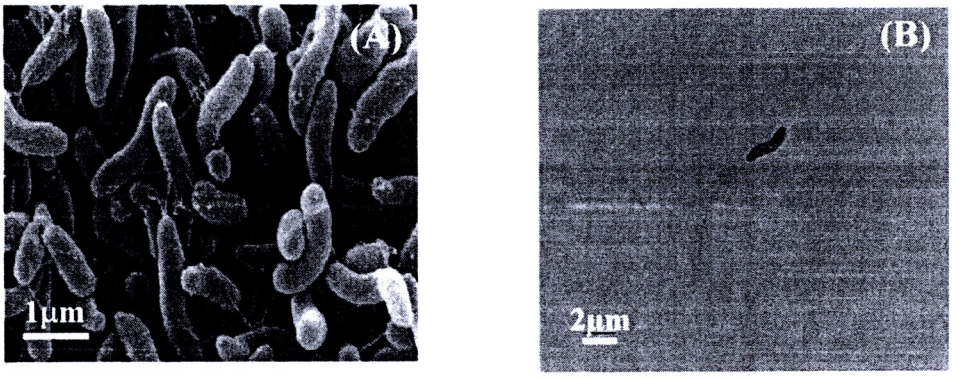

Figure 4.8 (A) Scanning electron micrograph of strain ND1-1 grew on JCM medium no. 377 at 37 °C. Bar, 1 μm., (B) Transmission electron micrograph of strain ND1-1 with the single polar flagellum. Bar, 2 μm.

ND1-1 grew under aerobic and anaerobic condition. Growth at pH range 5.0-9.0 (optimally at pH 8.0), at 10-47°C (optimally at 37°C) and in 1-22 % w/v NaCl (optimally in 9-10%, w/v NaCl) but no growth in the absence of NaCl. Positive for catalase, oxidase, hydrolysis of casein, gelatin, starch and L-tyrosine; nitrate reduction, Voges-Proskauer, arginine decarboxylase, acid phosphatase, alkaline phosphatase, cystine arylamidase, DNase, esterase lipase (C8), N-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase, leucine arylamidase, Napthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase but negative for hydrolysis aesculin and Tween 80; citrate utilization, α-chymotrypsin, esterase (C4), α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase (C14), α-

mannosidase, urease, H_2S production, methyl-red and indole formation. Acid was produced from D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, ribose, sucrose, trehalose and D-xylose but not from L-arabinose, cellobiose, *myo*-inositol, lactose, melibiose, melezitose, raffinose, rhamnose, salicin and sorbitol. Utilized L-alanine, crotonic acid, fumaric acid, glucoric acid, glycine, propionate, pyruvate, sodium glycolate, succinate, D-xylose and slight growth in sodium hipurate but not utilize L-arginine, L-arabinose, betaine, cellobiose, citric acid, D-fructose, galactose, glucose, ρ-hydroxybenzoic acid, lactose, sucrose, malonic acid, L-ornithine, sodium benzoate, sodium tartate and sorbitol. Strain ND1-1 was sensitive to ampicilllin (10 μg), choramphenicol (30 μg), nalidixic acid (30 μg), novobiocin (5 μg), rifampicin (30 μg) and tetracycline (30 μg) but resistant to bacitracin (10 units), gentamicin (10 μg), kanamycin (30 μg) and neomycin (30 μg).

ND1-1 contained Q-8 as the major respiratory lipoquinone. Major cellular fatty acids were as shown in Table 4.18. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) were the predominantant polar lipids (Figure 4.9). DNA G+C content was 49.0 mol%.

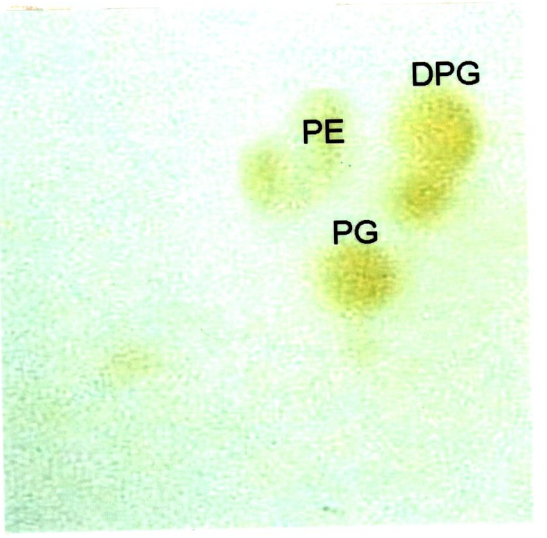

Figure 4.9 Thin-layer chromatogram of the total polar lipids of ND1-1

Comparison of 16S rDNA sequence of strain ND1-1 (1,487 nt) with other members of the family *Vibrionaceae,* phylogenetic tree (Figure 4.10) indicated that it was closed to members of the genus *Salinivibrio* showing 98.3% similarity to *S. costicola* subsp. *costcola* NCIMB 701^T, 98.3% to *S. costicola* subsp. *alcaliphilus* DSM 16359^T, 98.2 % to *S. costicola* subsp. *vallismortis* DSM 8285^T and 98.6 % to *S. proteolyticus* AF-2004^T(Table 4.19).

Additionally, the 16S rDNA of strain ND1-1 contained the signature nucleotides found in the genus *Salinivibrio* (Mellado et al., 1996) and its secondary structure at positions 178 to 197 (*Escherichia coli* 16S DNA genes sequence numbering) was more closed to that of *S. costicola* subsp. *vallismortis* DSM 8285^T, *S. proteolyticus* AF-2004^T (Figure 4.11) and *Vibro* species than those of *S. costicola* subsp. *costicola* NCIMB 701^T and *S. costicola* subsp. *alcaliphilus* DSM 16359^T as reported by Huang et al., 2000. In the primary sequence of the 16S rDNA of *S. costicola* subsp. *costicola* NCIMB 701^T and *S. costicola* subsp. *alcaliphilus* DSM 16359^T has insertions at helices between position 183 -193 and 207-214 (Figure 4.12) while ND1-1 absence.

Table 4.18 Cellular fatty acid composition of strain ND1-1 and related *Salinivibrio* species

Strains: 1, ND1-1; 2, *S. costicola* subsp. *costicola* JCM 15095^T; 3, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 4, *S. costicola* subsp. *vallismortis* JCM 15096^T; 5, *S. proteolyticus* DSM 19052^T.

Fatty acid[a]	Percentage of total[a]				
	1	2	3	4	5
Saturated straight-chain					
$C_{12:0}$	14.0	5.1	7.2	6.6	4.0
$C_{14:0}$	8.1	3.7	2.8	6.8	3.6
$C_{16:0}$	16.4	24.4	15.0	16.3	20.2
$C_{18:0}$	1.5	1.2	1.6	1.4	3.0
Unsaturated straight-chain					
$C_{16:1}\omega 9c$	2.1	4.3	3.1	3.7	3.7
$C_{18:1}\omega 5c$	-	-	-	-	1.2
$C_{18:1}\omega 7c$	10.3	14.3	11.8	21.7	27.3
$C_{18:1}\omega 9c$	1.2	1.6	1.8	2.8	4.2
Saturated branched-chain					
iso-$C_{13:0}$	-	-	4.6	1.4	-
iso-$C_{14:0}$	1.7	-	-	-	-
iso-$C_{15:0}$	-	-	1.3	1.9	-
iso-$C_{16:0}$	2.8	-	1.9	1.7	1.4
iso-$C_{17:0}$	-	-	2.0	1.2	-
Hydroxylated fatty acids					
$C_{12:0}$ 3OH	8.7	3.7	4.4	3.4	2.7
SF 2[b]	5.4	4.6	6.3	5.8	3.4
SF 3[c]	27.7	37.1	35.7	25.4	25.3

[a]Values are percentage of total cellular fatty acids.

[b]Summed feature 2 contains iso-$C_{16:1}$ and /or $C_{14:0}$ 3OH.

[c]Summed feature 3 contains $C_{16:1}\omega 7c$ and /or iso-$C_{15:0}$ 2OH.

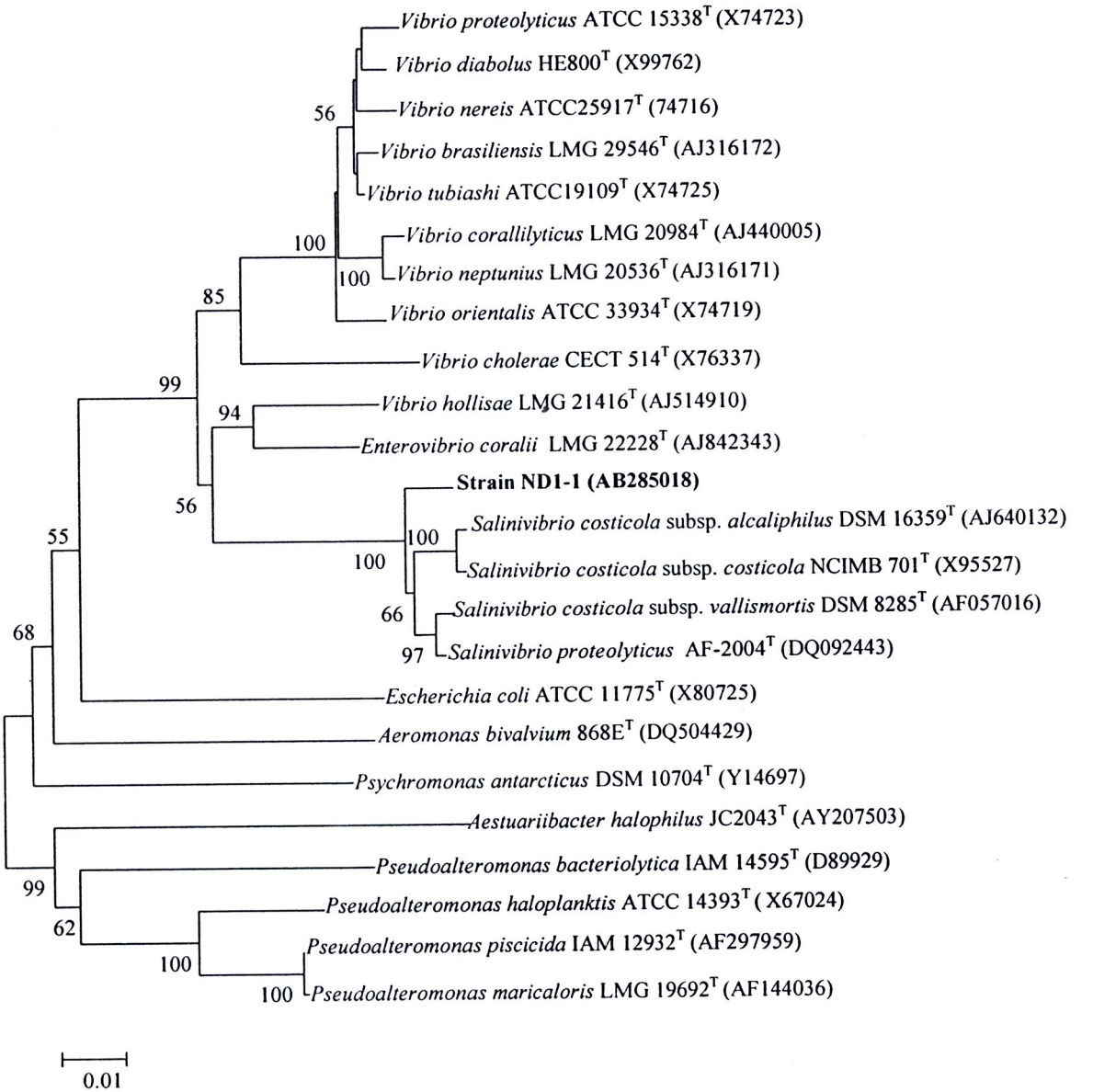

Figure 4.10 Phylogenetic tree showing the relationships between strain ND1-1^T, *Salinivibrio* species and related taxa based on 16S rDNA sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

Table 4.19 Percentage similarities of ND1-1 (Group VI) and related taxa.

		%Similarity																						
	Accession no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	AJ640132	100																							
2	X95527	99.7	100																						
3	AF057016	98.6	98.6	100																					
4	DQ092443	98.6	98.6	99.5	100																				
5	**ND1-1**	98.4	98.4	98.2	98.7	100																			
6	AJ514910	93.6	93.6	93.8	93.8	93.5	100																		
7	AJ842343	93.7	93.8	93.7	93.7	93.5	96.3	100																	
8	X74716	92.7	92.6	92.8	93.1	93.2	93.6	94.1	100																
9	X74725	92.8	92.8	93.1	93.5	93.5	93.9	94.5	99.3	100															
10	X74723	92.7	92.7	92.8	93.4	93.4	94.2	94.3	98.9	99.1	100														
11	X7471	92.9	93.0	93.0	92.8	92.9	94.5	94.7	98.1	98.7	97.9	100													
12	X99762	92.6	92.6	92.9	93.2	93.3	93.7	94.4	98.8	99.0	98.6	98.5	100												
13	AJ440005	92.0	92.0	92.1	92.1	92.3	94.2	93.7	98.2	98.5	97.7	98.0	97.7	100											
14	AJ316171	92.2	92.3	92.1	92.2	92.4	94.3	93.9	98.3	98.7	98.1	98.2	97.9	99.4	100										
15	AJ316172	92.8	92.8	92.9	93.3	93.6	93.9	94.5	99.1	99.5	98.8	98.5	98.9	98.4	98.4	100									
16	X76337	90.7	90.6	91.5	91.3	90.7	92.7	93.9	94.5	94.9	95.0	94.6	94.5	94.4	94.8	94.6	100								
17	X80725	88.2	88.2	88.0	88.2	88.1	90.0	90.6	90.0	90.3	89.8	89.9	90.0	90.0	90.2	90.3	90.8	100							
18	DQ504429	88.2	88.2	88.8	88.7	87.7	89.2	90.7	88.6	89.1	89.0	89.4	89.1	88.9	89.0	89.4	89.8	90.2	100						
19	AF297959	88.2	88.1	88.9	89.3	88.6	89.2	89.2	89.5	89.5	89.7	89.1	89.7	89.1	89.1	89.6	88.9	88.0	88.0	100					
20	AF144036	88.1	88.1	88.8	89.2	88.5	89.1	89.1	89.4	89.5	89.6	89.0	89.7	89.0	89.0	89.5	88.8	87.9	88.0	99.9	100				
21	X67024	88.4	88.4	88.8	89.3	88.9	88.6	89.3	88.3	88.7	89.1	88.2	88.7	87.8	88.2	88.8	88.5	87.7	87.5	96.4	96.3	100			
22	D89929	86.3	86.4	86.5	86.7	86.1	88.0	87.4	88.0	88.2	88.4	88.0	88.4	88.1	88.3	88.3	88.3	89.2	87.8	91.5	91.4	91.5	100		
23	AY207503	86.6	86.6	87.3	87.6	86.8	86.2	86.4	85.8	86.1	86.5	85.4	86.8	84.8	85.1	86.1	85.9	85.6	86.2	89.0	88.9	89.0	88.3	100	
24	Y14697	90.7	90.7	90.6	90.4	90.2	88.6	89.7	88.9	89.1	89.0	89.3	89.1	88.4	88.7	89.3	88.2	87.8	89.4	89.2	89.1	90.7	88.5	86.6	100

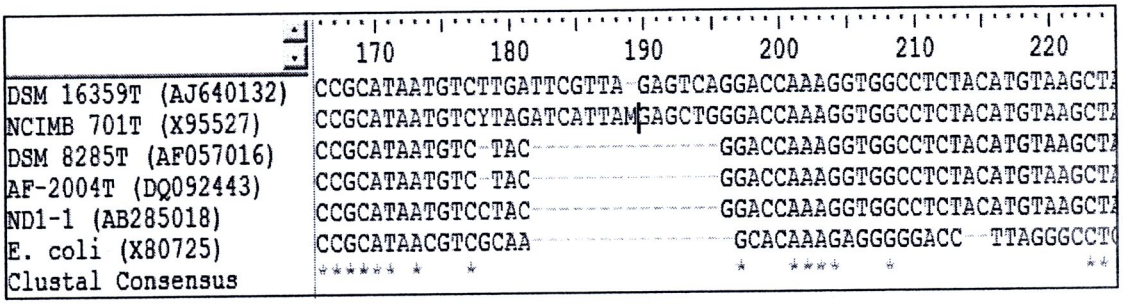

Figure 4.11 Alignment of 16S rDNA at positions 160 to 230 (*Escherichia coli* 16S DNA genes sequence numbering) of ND1-1 and related species.

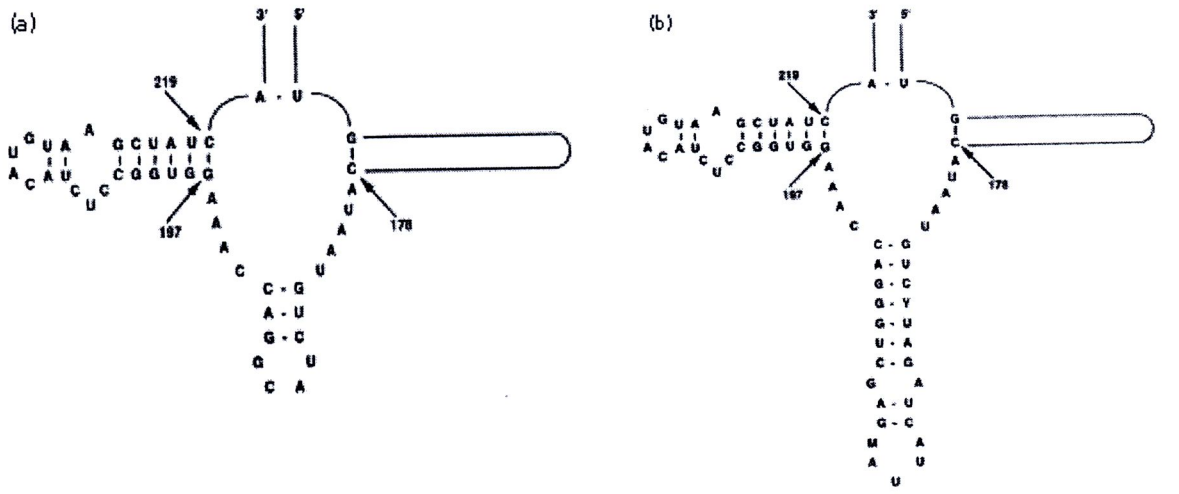

Figure 4 .12 Comparison of the secondary 16S rDNA structures from *S. costicola* subsp. *vallismortis* DSM 8285^T (a) and *S. costicola* subsp. *costicola* NCIMB 701^T (b). The numbers represent the two helices from positions 178 to 197 and From 197 to 219 (*E. coli* numbering according to Winker and Woese, 1991). (Huang et al., 2000).

Strain ND1-1 had low levels of DNA-DNA relatedness to *S. costicola* subsp. *costicola* JCM 15095^T (33.2%), *S. costicola* subsp. *alcaliphilus* DSM 16359^T (38.4%), *S. costicola* subsp. *vallismortis* JCM 15096^T (59.7%), and *S. proteolyticus* AF-2004^T (42.1%). Therefore, this strain should be the novel halophilic bacterium and name as *Salinivibrio siamensis* The differential characteristics of ND1-1 and *S. costicola* subsp. *costicola* JCM 15095^T, *S. costicola* subsp. *alcaliphilus* DSM 16359^T, *S. costicola* subsp. *vallismortis* JCM 15096^T and *S. proteolyticus* DSM 19052^T were summarized in Table 4.20.

Table 4.20 Differential characteristics of ND1-1 and related *Salinivibrio* species

Strains: 1, ND1-1; 2, *S. costicola* subsp. *costicola* JCM 15095^T; 3, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 4, *S. costicola* subsp. *vallismortis* JCM 15096^T; 5, *S. proteolyticus* DSM 19052^T. Data were taken from this study and from Huang et al., 2000, Romano et al., 2005, Amoozegar et al., 2008.

Characteristic	1	2	3	4	5
Colony colour	Cream	Cream	Cream pink	Cream white	Cream white
Flagellation	One polar flagellum	One polar flagellum	ND	One or two polar flagella	One polar flagellum
NaCl range (%, w/v)	1-22	2-18	2-22	0-12.5	1-17
Optimum growth in NaCl (%)	9-10	10	9	2.5	5
Temperature range (°C)	10-47	15-45	10-45	20-50	10-45
Optimum temperature (°C)	37	34	37	37	32-35
pH range	5-9	6-9	6-10	5.5-8.2	5-9.5
Optimum pH	8	8	8	7.3	8-8.5
Voges-Proskauer	+	+	-	+	+
Nitrate reduction	-	-	+	-	-
Esterase (C4)	-	+	-	+	w
Naphthanol-AS-BI phosphohydrolase	+	+	-	w	-
N-acetyl-β-glucosaminidase	+	+	-	+	w
Hydrolysis of : Aesculin	-	-	+	-	-
Starch	+	-	-	+	+
L-Tyrosine	+	+	w	-	-
Acid from: D-Cellobiose	-	-	+	+	-
D-Xylose	+	-	-	-	+
Utilization of: D-Cellobiose	-	+	-	+	-
Crotonic acid	+	-	+	-	ND
D-Fructose	-	-	-	+	+
Glycine	+	-	+	-	+
Malonic acid	-	-	+	-	+
Succinate	+	-	+	-	+
D-Xylose	+	-	-	-	-
G+C content (mol%)	49	49.9 or 50.0	49.3	50.0	49.5
Source of isolation	Fermented fish	Cured meat	Saltish spring	Death valley	Hypersaline lake

+, Positive; w, weakly positive; -, negative; A, aerobe; FA, facultative anaerobe; ND, no data.

4.2.6 Group IX

Group IX contained isolate R5-7. Cell size was approximately 0.6-0.8 x 2.5-5 μm. Colonies were cream in colour (0.3 –3 mm diameter) after 3 days of incubation at 37°C on JCM no. 377 medium. R5-7 isolate grew over a wide range of salinity between 1 and 25% w/v, with optimal growth at 10% and maximum temperature for growth was 45°C. Utilized L-arabinose, betaine, ρ-hydroxybenzoic acid, citric acid, D-fructose, fumaric acid, galactose, sodium glycolate, pyruvate, malonic acid, succinate, , sorbitol, sodium benzoate L-arginine, L-ornithine and L-alanine, but not crotonic acid, glucoric acid, glycine, D-xylose, cellobiose, glucose, lactose, propionate, sucrose, sodium hipurate and sodium tartate. The phenotypic characteristics and other properties of R5-7 isolate was summarized in Table 4.21.

R5-7 isolates contained *meso*-DAP as in the cell wall peptidoglycan. The predominant respiratory ubiquinone (Q) found in the R5-7 isolate was Q-9. The major fatty acids profile of R5-7 and *C. salexigens* KTCC 12941^T were shown in Table 4.22. The DNA G-C content was 64 mol%. On the basis of 16S rDNA sequence and phylogenetic analyses, R5-7 isolates (1,499 nt) indicated that it was clustered within the family *Halomonadaceae* and in the genus *Chromohalobacter* (Figure 4.13). The isolate R5-7 was closely related to *C. salexigens* KTCC 12941^T. It exhibited sequence similarity values of 98.8% with *C. salexigens* KTCC 12941^T and *C. israelensis* ATCC 43985^T, respectively (Table 4.23). Isolate R5-7 showed high DNA-DNA relatedness with *C. salexigens* KCTC 12941^T (98.7%) (Table 4.24). In addition, their phenotypic characteristics and fatty acid profiles of R5-7 was agreed with that of the genus *Chromohalobacter* (Aral et al., 2001). Based on the results mentioned above and phenotypic properties indicated that isolate R5-7 isolate was identified as *C. salexigens*.

Table 4.21 Phenotypic characteristics of Group IX (R5-7) and *Chromohalobacter salexigens* KCTC 12941^T. Data in this study.

Characteristics	R5-7	KCTC 12941^T
Cell shape	Rods	Rods
Temperature range (° C)	10-45	10-45
NaCl range (%)	3-25	0.9-25
pH range	6-9	5-10
Oxidase	+	+
Catalase	+	+
Nitrate reduction	+	+
Citrate synthase	+	+
H_2S production	-	-
Urease	-	-
Hydrolysis of		
Casein	-	-
Esculin	-	-
Gelatin	-	-
Starch	+	+
Tyrosine	-	-

+, Positive; -, negative

Table 4.22 Cellular fatty acid composition of R5-7 and *C. salexigens* KCTC 12941^T.

Fatty acid	Percentage of total[a]	
	R5-7	KCTC 12941^T
Saturated straight-chain		
$C_{10:0}$	3.7	7
$C_{12:0}$	5.9	10.0
$C_{16:0}$	25.3	21.0
Unsaturated straight-chain		
$C_{16:1}$ *ω5c*		6.0
$C_{18:1}$ *ω7c*	13.5	23.8
$C_{17:0}$ cyclo	7.0	2.7
$C_{19:0}$ cyclo *ω8c*	29.7	
Hydroxylated fatty acids		
$C_{12:0}$ 3OH	8.0	13.4
SF 3[c]	3.2	4.8

[a]Values are percentage of total cellular fatty acids.

[c]Summed feature 3 contains $C_{16:1}$ *ω7c* and /or iso-$C_{15:0}$ 2OH.

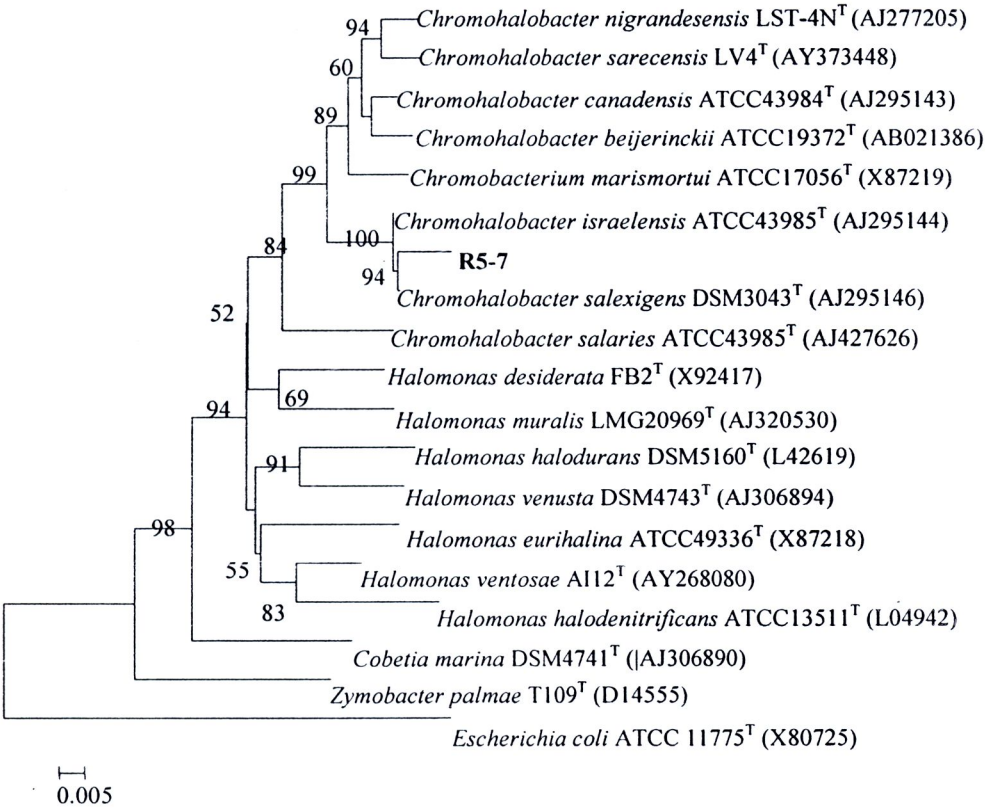

Figure 4.13 Phylogenetic tree showing the relationships between strain R5-7,*Chromohalobacter* species and related taxa based on 16S rDNA sequences.The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 50%, based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

Table 4.23 Percentage similarities of R5-7 (Group IX) and related taxa.

	Accession no.	% Similarity																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	**R5-7**	100																		
2	AJ277205	95.9	100																	
3	AY373448	95.6	98.5	100																
4	AJ295143	95.7	98.4	98.6	100															
5	AB021386	95.9	97.4	97.7	98.7	100														
6	X87219	95.5	97.0	97.0	98.2	97.9	100													
7	AJ295144	98.8	97.1	96.9	97.0	97.2	96.7	100												
8	AJ295146	98.8	97.0	96.7	96.8	97.1	96.6	99.9	100											
9	X92417	92.9	93.3	93.1	93.5	93.3	94.7	94.1	93.9	100										
10	AJ320530	93.6	93.7	93.3	93.5	93.3	94.5	94.7	94.6	95.5	100									
11	L42619	92.0	93.8	93.6	94.2	93.3	92.8	93.2	93.0	94.1	93.2	100								
12	AY268080	93.4	94.4	94.3	95.0	94.1	94.5	94.6	94.4	95.5	94.3	94.8	100							
13	AJ306894	92.3	92.9	92.9	93.7	94.0	93.4	93.5	93.4	95.1	93.7	95.5	94.7	100						
14	X87218	93.0	94.7	94.7	94.8	93.8	93.6	94.1	93.9	93.9	93.3	93.8	95.7	93.1	100					
15	L04942	91.4	93.1	93.2	93.6	92.7	93.2	92.8	92.6	93.0	92.5	93.8	95.7	93.4	93.1	100				
16	AJ306890	91.6	92.2	92.0	92.9	92.2	91.6	92.9	92.8	92.4	92.6	92.9	93.6	92.6	93.0	92.2	100			
17	D14555	89.8	89.6	89.7	90.0	90.2	90.7	91.2	91.0	91.8	91.3	89.5	91.9	90.3	90.1	90.3	92.4	100		
18	X80725	82.5	82.3	82.1	82.3	82.8	82.4	83.9	83.8	83.8	83.0	82.5	82.8	83.9	82.0	82.3	83.4	84.5	100	
19	AJ427626	94.2	94.5	94.9	95.4	95.3	96.0	95.5	95.3	94.7	94.9	92.6	94.2	94.2	93.3	92.2	93.2	92.0	83.2	100

Table 4.24 DNA-DNA relatedness of the Group XI isolate and KCTC 12941^T

Isolate/ strain	% of DNA-DNA relatedness with label strains	
	R5-7	KCTC 12941^T
R5-7	100	98.7
C. salexigens KCTC 12941^T	94.8	100

4. 3 Protease producing halophilic bacteria

4.3.1 Screening of protease-producing halophilic bacteria

Total of 57 halophilic bacteria were isolated from 46 samples of pla-ra collected from markets and a home made factory (Table 4.24). Fifty-four isolates of halophilic bacteria showed proteolytic activity in the presence of 5% w/v NaCl concentration except the isolates, MS3-4 (Group IV), TP2-8 Group V) and R5-7 (Group IX) could not produce proteolytic activity. All of the isolates in Group I that identified as *V. dokdonensis* could produce proteolytic activity weakly (diameter of clear zone was $\leq$ 0.5 mm) in the presence of 10% w/v NaCl while almost of the isolates identified as *V. halodenitrificans* (Group II) and *V. marismortui* (Group II) produced moderately proteolytic activity (diameter of clear zone was 0.5-10 mm). All of *Halobacillus* species (Group VI) isolates exhibited strongly protease except strain HR1-1. For the isolates of halotolerant *Bacilus* species (Group VII) produced strongly clear zone in the presence of 5% w/v NaCl, however they also produced proteolytic activity in 10% w/v NaCl when incubated for long time of the cultvation. From these result, the salt requirement for optimum enzyme secretion varied significantly among the isolates. The salt dependency was, however, relatively lower when compared to the moderately halophilic group.

From the previous research, the protease-producing halphilic bacteria were isolated from fermented fish in Thailand. There are *Fillobacillus* sp. RF2-5 (Hiraga et al., 2005), *Halobacillus* sp. SR5-3 (Sirilak et al., 2006), *Vigibacillus* sp SK37 (Sinsuwan et al., 2007), and *Virgibacillus marismortui* NB2-1 (Chamroensaksri

et al., 2008). These protease-producing halophilic bacteria were Gram-positive bacteria and theirs protease were characterized. In this study, a Gram-negative curved rods, strain ND1-1 was selected for further study due to its novelty and high protease production.

Table 4.25 The caseinolytic halo-forming colonies of isolates

Strain	NaCl concentration (% w/v)	
	5%	10%
Group I :		
MSK2-1, BKL5-3	+++	+
BKL4-2, LB3-2, MSK1-1	++	+
CHM2-2, MSK3-1	+	+
CHN1-1, CHN2-2, TP2-1	+	-
Group II :		
CHM1-4, BKL6-1,BKL1-1,TP2-6 TSY2-3,BSR3-1	+++	+++
TP4-3	+++	++
TP3-1, TSR2-1	++	++
BKL2-2	++	+
CHM1-3, PT5-1	+	+
AY1-2	+	+
Group III:		
SB2-2	+++	+++
BSR4-1	+++	++
PR2-2	+	+++
TSR5-2	++	+++
BY2-4, BT3-1, PR2-1, TM4-3	++	+
TP1-3	++	+
RM1-1, ND2-1	+	++
BKS1-1, RM2-1	+	+
Group VI:.		
AG1-3, TPA3-2, AG2-2, BY1-1,MS2-6	+++	+++
AG2-2	++	+++
HR1-1		
Group VII:		
N20-1	+++	++
TP1-1, MS1-3, MS3-2, NS1-7, TP4-4, TP4-6, TP1-7, TP4-7, PJ1-6	+++	+
Group VIII:		
ND1-1	+++	+++

+++, strong; ++, moderate; +, weak activity; -, no activity.

4.3.2 Protease production and the effect of various parameters

4.3.2.1 The kinetic of growth and protease production.

The kinetic of growth and protease production were investigated. The lag phase of bacterial growth was 4 h and after 28 h the bacterial growth reached to the stationary phase. No extracellular protease activity was shown during the 0-4 h (lag phase) and protease production started by the middle of exponential phase. The maximum protease production of ND1-1 in medium JCM no. 377 was determined in the stationary phase. However, the production was decreased during prolonged cultivation. (Figure 4.14).

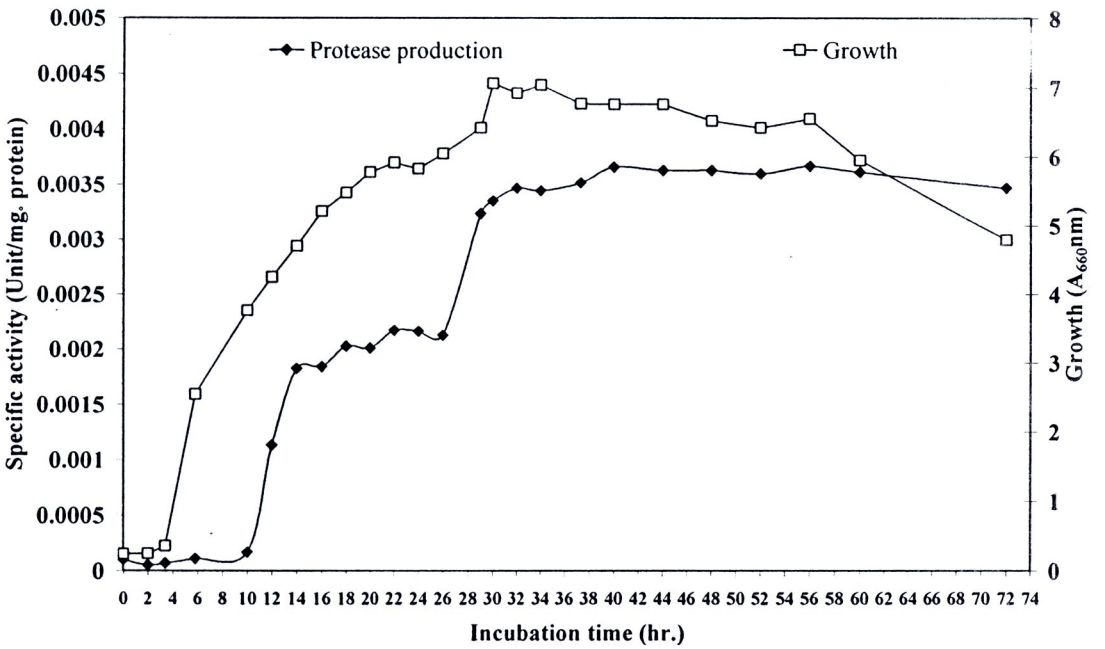

Figure 4.14 The kinetic of growth and protease production of strain ND1-1 in the JCM no. 377 medium.

4.3.2.2 Optimization of crude protease production

Optimization of crude protease production of the protease producing strain ND1-1 was carried out in the JCM no. 377 medium. The influence of several factors, medium composition, NaCl concentration, initial pH and temperature on protease production was studied. An effective prior condition was used as the basis in the latter experiment until the optimal condition was obtained.

4.3.2.2.1 Effect of different nutrient on protease production: The production of the protease was strongly influenced by the composition of the culture medium (Table 3.2). Protease production was analysed when casamino acids in culture medium was omitted and replace by 0.5% w/v other nitrogenous nutrient. Among nitrogenous nutrient tested, maximal protease production (0.0176 Units) was obtained when 0.5% w/v skim milk was used in (Figure 4.15). This result indicated that protease production was highly repressed by the presence of casamino acids. The presence of 0.5% yeast extract and 1-2% w/v gelatin stimulated growth of ND1-1 but not protease production. Lama et al (2005) reported that *Salinivibrio* sp 18AG^T produced highest protease containing 12% w/v gelatin. To optimize the protease production of ND1-1, the skim milk concentration added into modified medium JCM no. 377 (without casamino acids) was varied. Maximal protease production was obtained at 0.5% w/v skim milk. Higher concentration of skim milk resulted in higher growth (Figure 4.16).

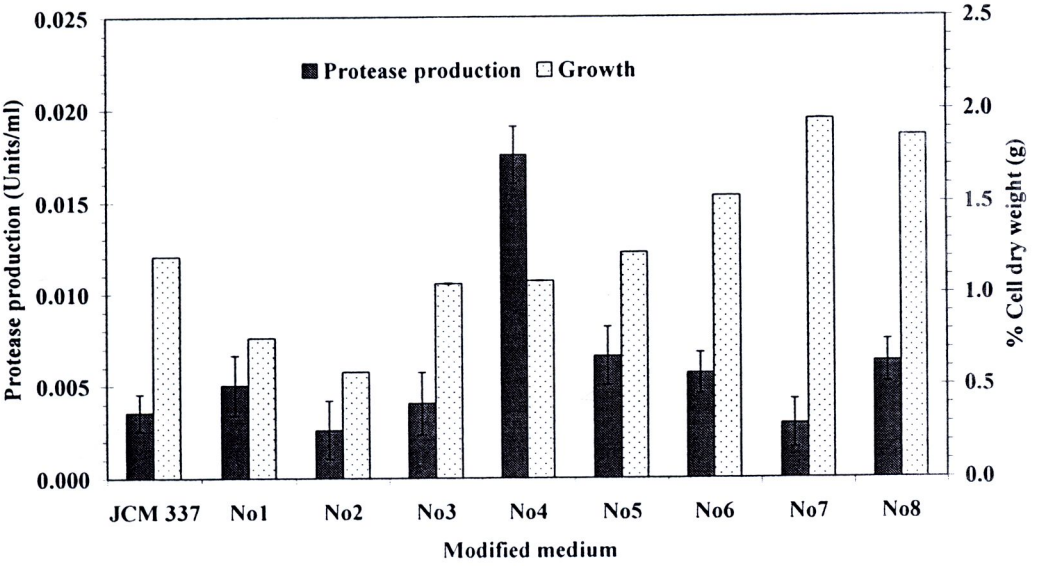

Figure 4.15 Effect of different nutrient on protease production. No.1; JCM no. 377 without casamino acids, No.2; JCM no. 377 without yeast extract, No. 3- No. 8; Modifed JCM no. 377 by substitution of casamino acids with 0.5%yeast, 0.5% skim milk, 0.5% gelatin,1% gelatin, 2% gelatin and 0.5% soy protein respectively. The sample was taken after 48 h. of incubation.

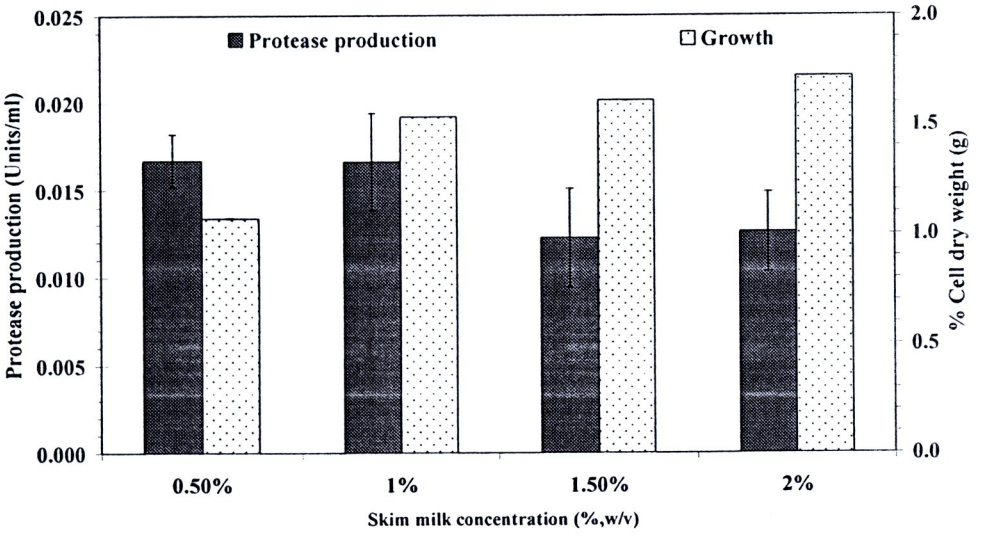

Figure 4.16 Effect of skim milk concentration in modified JCM no. 377(without casamino acids) on protease production and growth of strain ND1-1.

4.3.2.2.2 Effect of NaCl concentration on protease production: The strain ND1-1 was cultivated in modified JCM no. 377 medium containing 0.5% skim milk (No. 4) and 0, 5, 10, 15 or 20% w/v of NaCl , pH 8.0 and incubated with shaking (200 rpm) at 37°C for 5 days. The result was shown in Figure 4.17. The optimal concentration of NaCl for both protease production and growth was 10% w/v. Maximum protease production was obtained in the modified medium containing 10% w/v NaCl after 3 days of incubation. The strain ND1-1 was not able to grow in the absence of NaCl indicated that it was a moderate halophilic bacterium according to the definition of Kushner (1985).

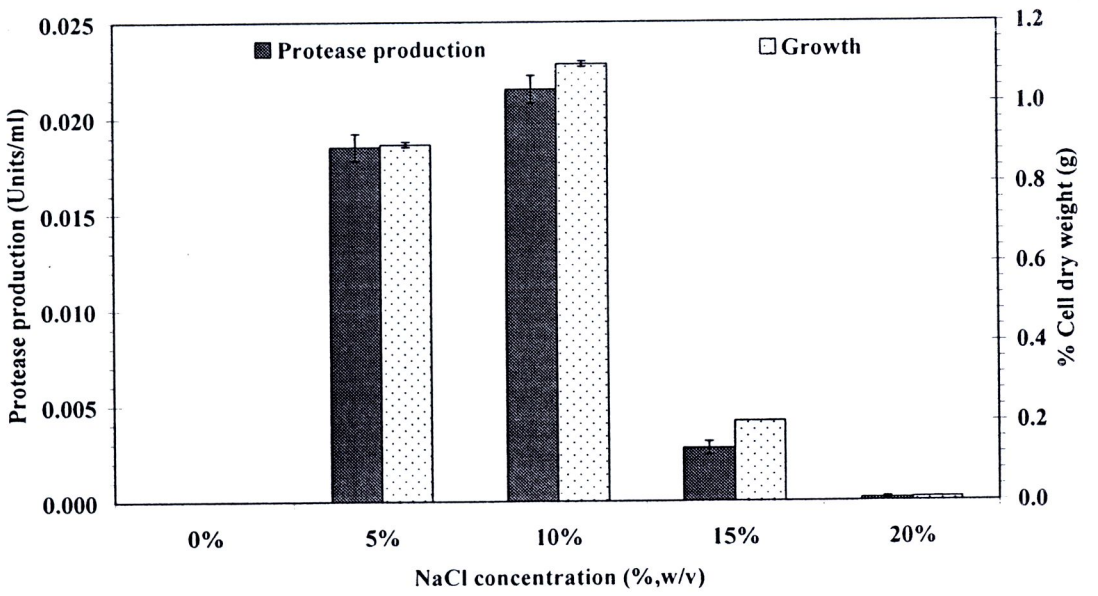

Figure 4.17 Effect of NaCl concentration on protease production and growth of strain ND1-1.

4.3.2.2.3 Effect of initial pH on protease production: The strain ND1-1 was cultivated in modified JCM no. 377 medium containing 0.5% w/v skim milk and 10% w/v NaCl which was adjusted to pH 5.0, 6.0, 7.0, 7.5, 8.0, 9.0, or 10 ; and incubated at 37°C under shaking condition (200 rpm) for 2 days. As shown in Figure 4.18 the optimal pH for protease production and growth was 8.0. The strain ND1-1 grew at pH 5.0 to 9.0. There was no growth and protease production at pH 10.0.

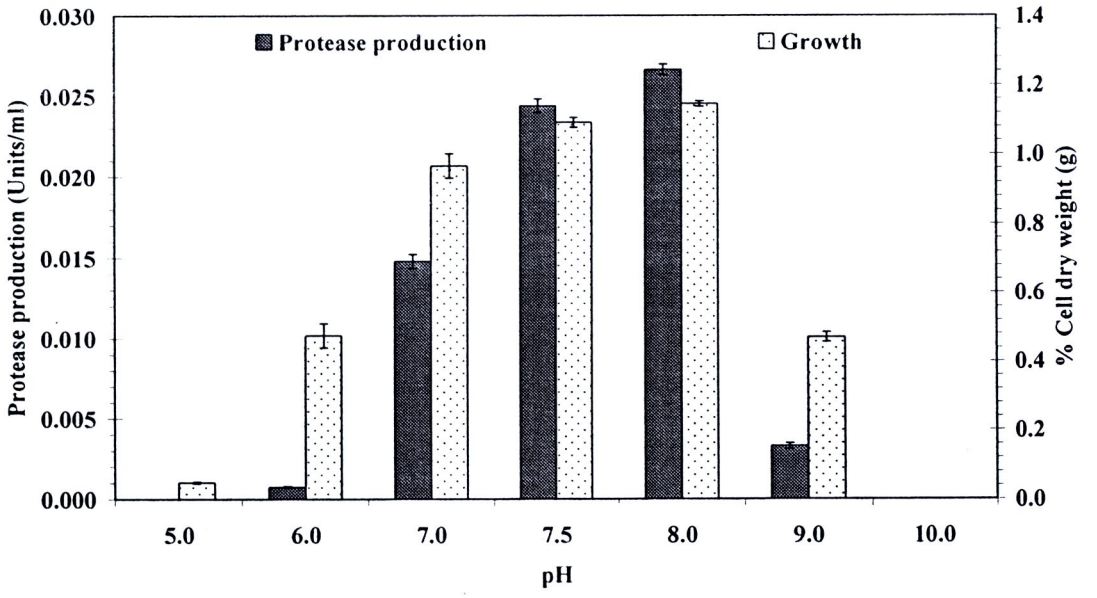

Figure 4.18 Effect of pH on protease production and growth of strain ND1-1.

4.3.2.2.4 Effect of incubation temperature on protease production

The strain ND1-1 was cultivated in modified JCM no. 377 medium containing 0.5% w/v skim milk, 10% w/v NaCl, pH 8.0 and incubated with shaking (200 rpm) at 25, 30, 37 40 or 45°C for 2 days. The result was shown in Figure 4.19 the optimal temperature for both protease production and growth was 37°C.

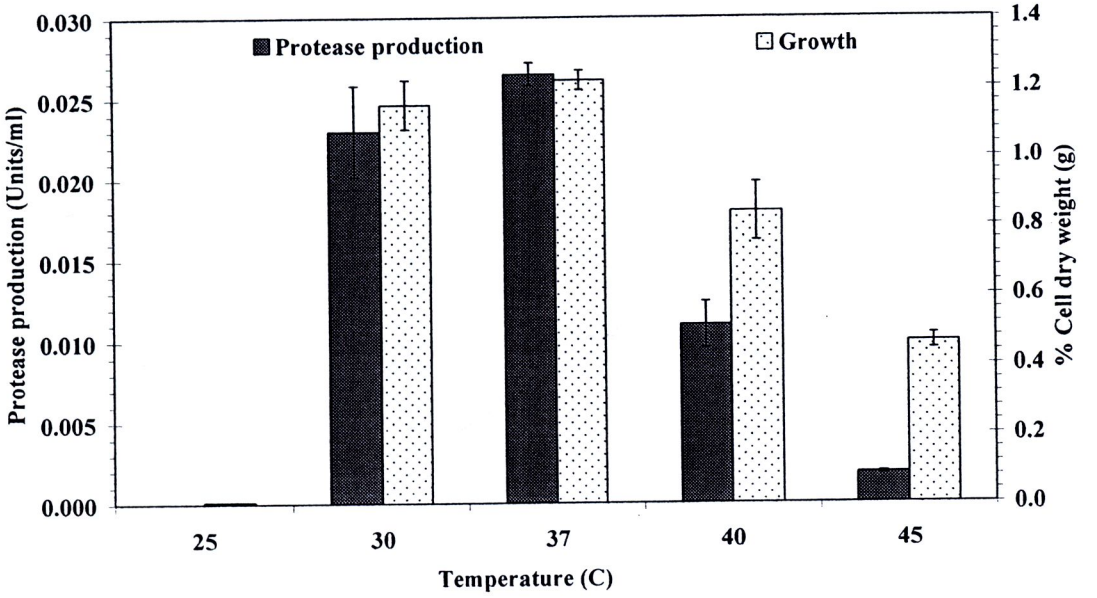

Figure 4.19 Effect of temperature on protease production and growth of strain ND1-1.

The protease production was strongly influenced by the composition of the culture medium in which the replacement of casamino acids with 0.5% skim milk. But yeast extract significantly repressed protease production and growth when omitted from JCN no. 377 medium. Maximum protease production occurred in the modified JCM no.377 medium in which casamino acids was omitted and replaced with 0.5% w/v skim milk, and incubated at 37°C with shaking (200rpm) for 2 days containing 10% NaCl, pH 8.0 with incubation at 37°C for 2 days. At optimal condition, crude protease produced by strain ND1-1 increased 6.25 times.

4.3.3 Preparation of purified enzyme for characterize

Purification step of ND1-1 protease is summarized in Table 4.26. After The culture filtrate was concentrated by ultrafiltration technique with 10,000 MWCO, total activity (886.3 units) of approximately 91% remained, while 9% of total protein was removed. From this result, purity of 14.3 fold was achived after ultrafiltration. After loading the concentrated filtrate onto anion exchanger, HiTrap Q XL column, the column was eluted by a 0-2.0 M NaCl gradient. Four protein peaks (A_{280}) was separated which an activity peak was eluted at 0.56-0.84 M NaCl (Figure 4.20). Large amount of proteins was removed after elution with 0-0.2 M NaCl, leading to an increase in purity fold of 223.0. Fractions with activity were pooled (Fraction no. 24-30). Specific activity of the pooled fractions increased to 66.9 units/mg protein.

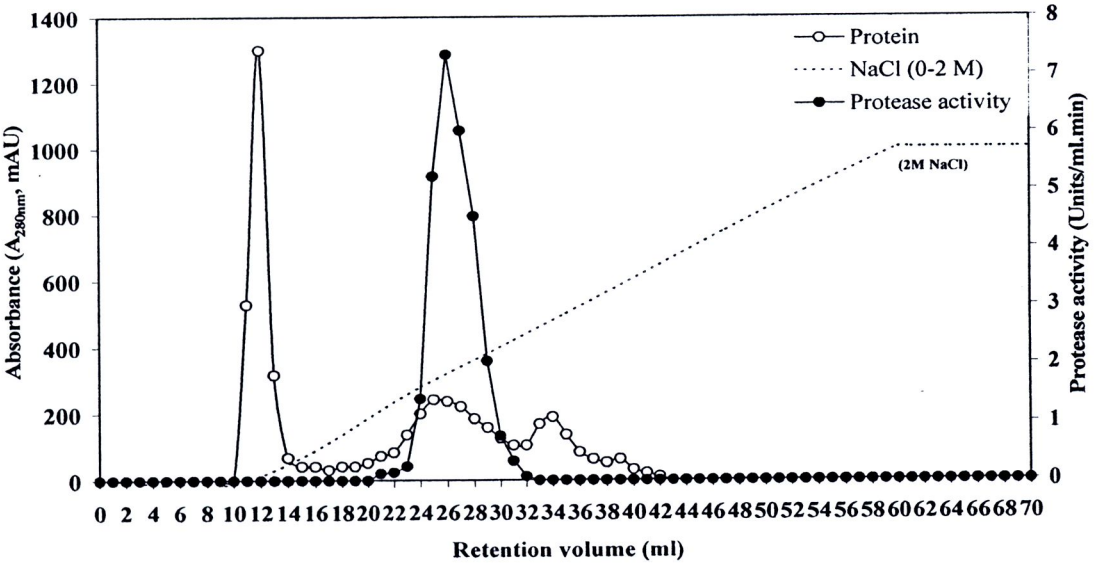

Figure 4.20 Elution profile of strain ND1-1 protease on HiTrap Q XL column. Elution was carried out with a gradient of 0 to 2.0 M NaCl in 50 mM Tris HCl, pH 8.0. Fractions of 0.5 ml were collected.

The pooled active HiTrap Q XL fractions were further purified by a size exclusion chromatography using Superose 12. The column separated protease enzyme from other proteins by molecular size (Figure 4.21).This step effectively separated protease from other protein contaminants. Purity of protease increased 258.3 folds with a yield of 8.3 % in this step.

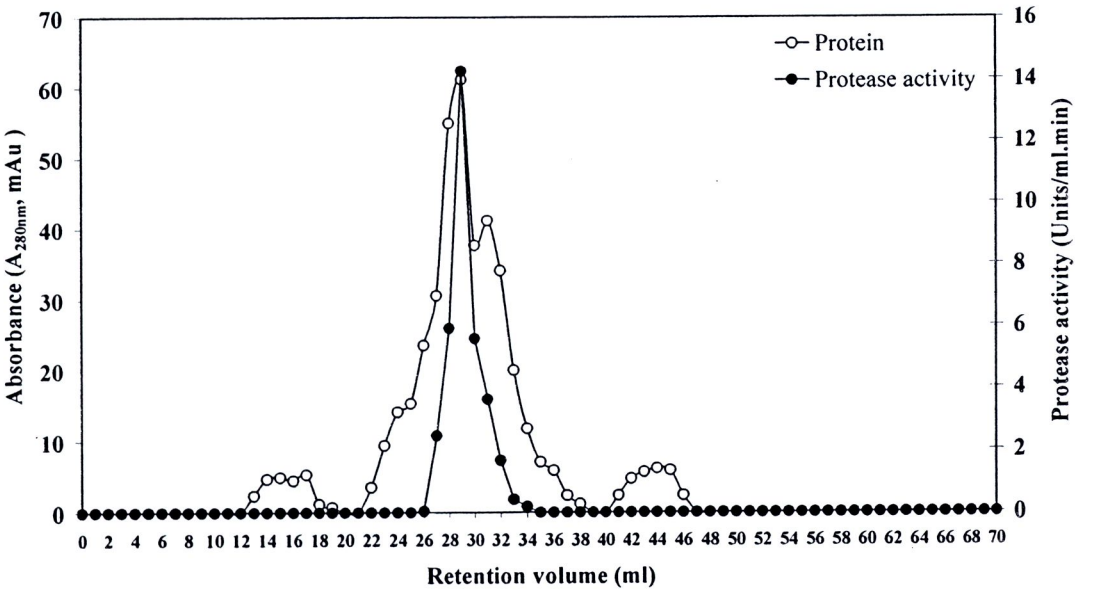

Figure 4.21 Elution profile of strain ND1-1 protease on Superose 12 column. Elution was carried out with 0.15 M NaCl in 50 mM Tris-HCl, pH 8.0 at a flow rate of 0.1 ml/min. Fractions of 0.5 ml were collected.

Table 4.26 Preparation of purified enzyme for characterization

Purification steps	Total activity (Units*)	Total protein (mg**)	Specific activity (Units/mg)	Purity (Fold)	Yield (%)
Crude extract	973.8	3262.8	0.3	1.0	100
Ultrafiltration	886.3	205.2	4.3	14.3	91.0
HiTrap Q XL	124.9	1.9	66.9	223.0	12.8
Superose 12	80.4	1.0	77.5	258.3	8.3

*The unit of enzyme activity is expressed as the μmoles of tyrosine per min.
**Protein concentration was measured by Lowry method.

The purity of the purified protease enzyme from ND1-1 was evaluated by native gel electrophoresis, followed by activity staining (Figure 4.22A and 4.22B). Protein pattern of concentrated ND1-1 protease during purification process is also shown in Figure 4.22A. The concentrated filtrate contained variety of proteins with different molecular weights (lane 1). After subjected to HiTrap Q XL column (lane 2), some proteins bands disappeared which it was removed by gradient below 0.56M NaCl or above 0.84 M NaCl. After Superose 12 chromatography, proteins with MW of higher than and lower than ND1-1 protease were removed by size exclusion. Finally, only one single protein band on native gel electrophoresis was obtained (Figure 4.22A). The single protein band position was agreed well with the activity band observed on activity staining gel (Figure 4.22B)

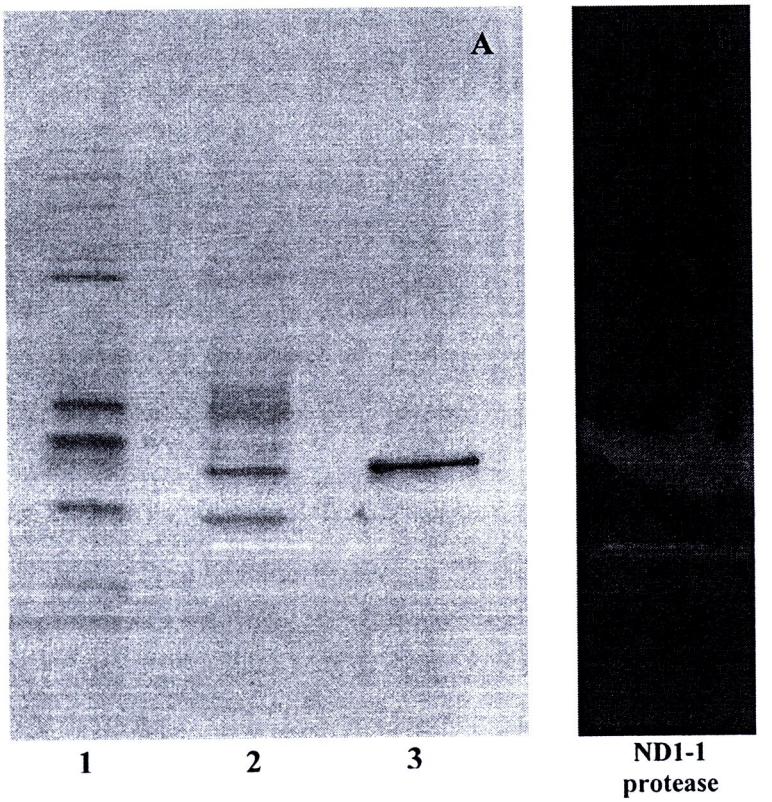

Figure 4.22 Protein pattern (A) and activity staining (B) of the purified protease from ND1-1 on native gel electrophoresis and protease activity staining at pH 8.0, 55°C. Lane 1, crude enzyme extract; lane 2, HiTrap Q XL fraction; lane 3, Superose 12 fraction (fraction no.29). Approximately same amount (15 μg) of protein on each lane was applied to 12.5% polyacrylamide gel and electrophoresis was carried out for 2 h at 15 mA per gel. After electrophoresis, the left-hand gel (A) was protein stained with Coomassie brilliant blue R-250 for protein and the right-hand gel (B) was activity stained by the method described in materials and methods.

4.3.4 Characteristics of the purified protease from ND1-1

4.3.4.1 Molecular mass of protease from ND1-1

By Superose 12 gel filtration chromatography, the molecular mass of the purified ND1-1 protease was 36.8 kDa. (Figure 4.23). SDS-PAGE analysis showed that the purified enzyme migrated as a single band with the molecular weight of 38.0 kDa (Figure 4.24). This result indicated that ND1-1 protease was monomeric protein with molecular weights of 36.8 (gel filtration method) and 38.0 (SDS-PAGE). The molecular weight of ND1-1 protease was closely to serine-metalloprotease from *Salinivibrio* sp. AF2004 (31kDa from SDS-PAGE, 29 kDa from gel filtration method); zinc-metalloprotease from *Salinivibrio* sp. AF2004 (38 kDa) and serine protease from *Salinivibrio* sp. strain 18AG^T (38 kDa). (Karbalaei-Heidari et al., 2007; Karbalaei-Heidari et al., 2007. and Lama, et al. 2005).

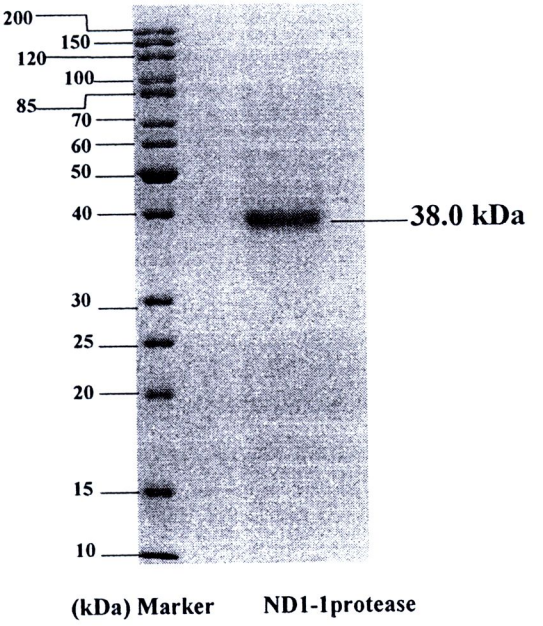

Figure 4.23 SDS-PAGE pattern (with reducing agent; 2% β-mercaptoethanol) of the purified protease from ND1-1 (fraction no.29 of Superose 12 column). Purified protease from ND1-1 (15 μg of protein) was applied to 12.5% polyacrylamide gels and electrophoresis was carried out for 2 h at 15 mA per gel. After electrophoresis, the gel was protein stained with Coomassie brilliant blue R-250 for protein. Positions of molecular mass markers are shown to the left. The positions of the purified protease from ND1-1 are indicated to the right.

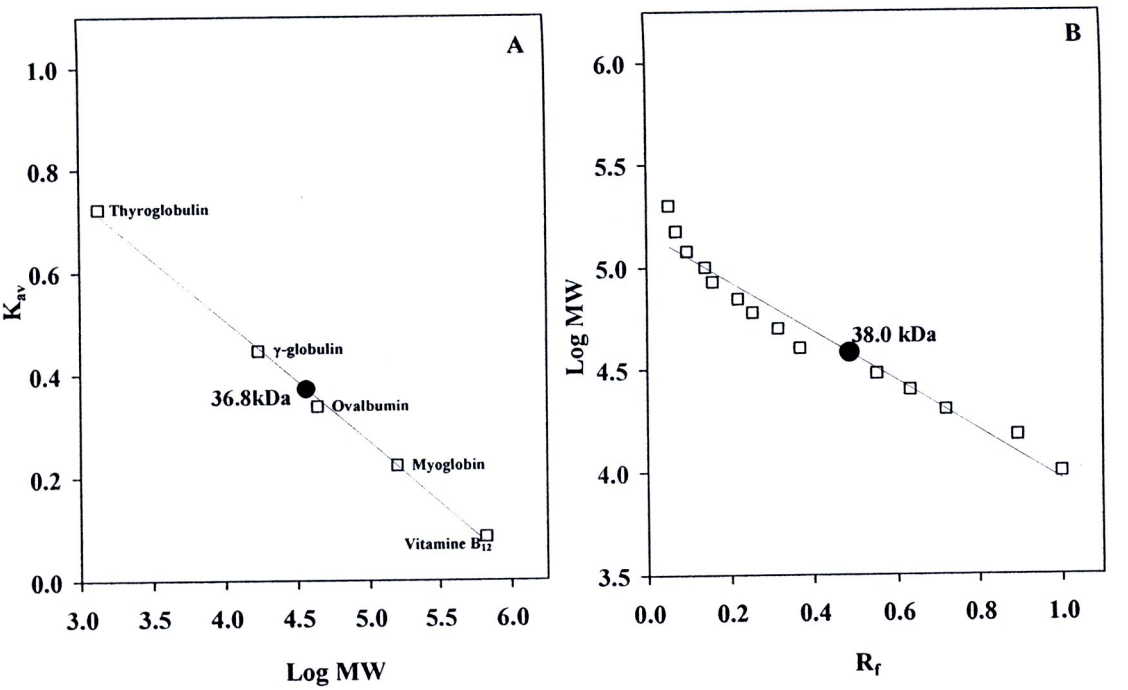

Figure 4.24 Calibration curve for the molecular weight determination on Superose 12 10/300 chromatography. (A) and SDS-PAGE with reducing agent (2% mercaptoethanol) (B) of the purified protease from ND1-1. •, purified protease from ND1-1.

4.3.4.2 Optimal temperature and thermal stability

An effect of optimal temperature and temperature stability on the purified ND1-1 protease activity is presented in Figure 4.25. The protease underwent thermal activation above 45°C with a maximum activity at 55°C and inactivated above 65°C. In addition, protease from ND1-1 was stable at 30-50°C which higher than 50% of its maximal was retained. The activity and stability which decreased drastically at high temperature, possibly due to the partial unfolding of the enzyme molecule.

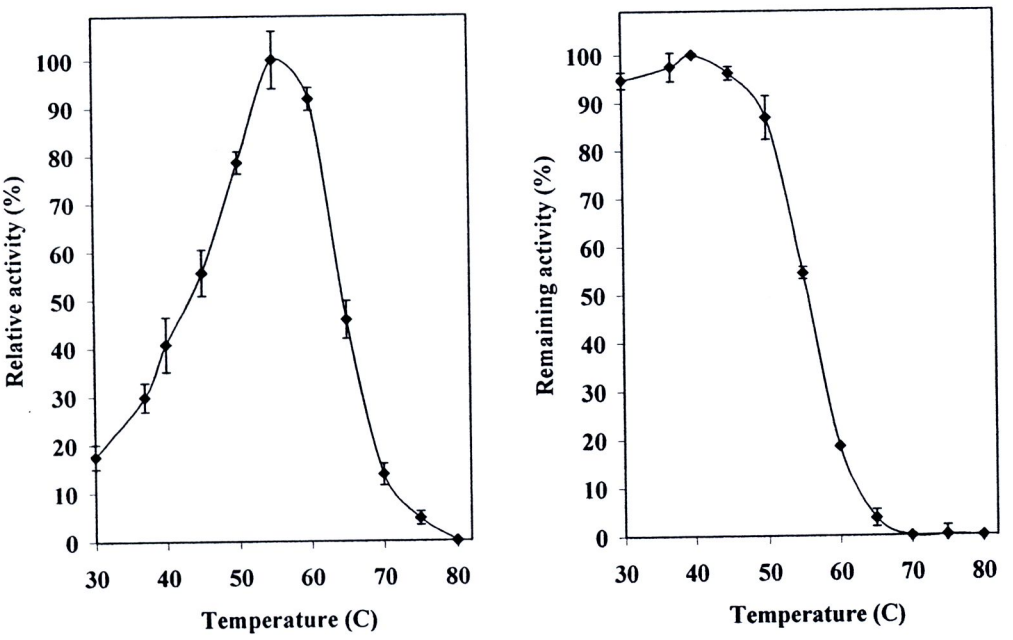

Figure 4.25. The effect of temperature on protease activity (A) and stability (B) of the purified protease from ND1-1. Means of triplicate determinations are presented, and bars indicate standard deviation. Absence of bars indicates that errors are smaller than symbols. Optimum activity (A) was analyzed using casein as a substrate for 1 h, pH 7.5 at various temperature For the stability test (B), the enzyme was incubated at various temperature for 1 h in 50 mM Tris-HCl buffer (pH 7.5) and then cooled on ice. Then remaining activity was analyzed using casein as a substrate pH 7.5 at 37°C for 1 h was set as 100%.

4.3.4.3 Optimal pH and pH stability

An effect of optimal pH and pH stability on the purified protease from ND1-1 is shown in Figure 4.25. The activity was highest (2.2 Units) at pH 8.0, there was considerable loss of activity at pH ≤7.0 and at pH ≥8.0. No activity detected at pH ≤ 4. The enzyme was stable in narrow pH range (pH 7.0-9.0). From this results the purified ND1-1 protease might undergo denaturation at at pH <7.0 and at pH > 9.0, where the enzyme conformation changed. Most of protease from moderately halophilic bacteria showed optimal pH activity and pH stability in broad alkaline pH range, such as protease from *Fillobacillus* sp. RF2-5 (Hiraga et al., 2005), *Halobacillus* sp SR5-3 (Namwong et al., 2006), *Salinivibrio* sp. AF2004 (Amoozegar et al., 2006) which in contrast with the results obtained from ND1-1 protease.

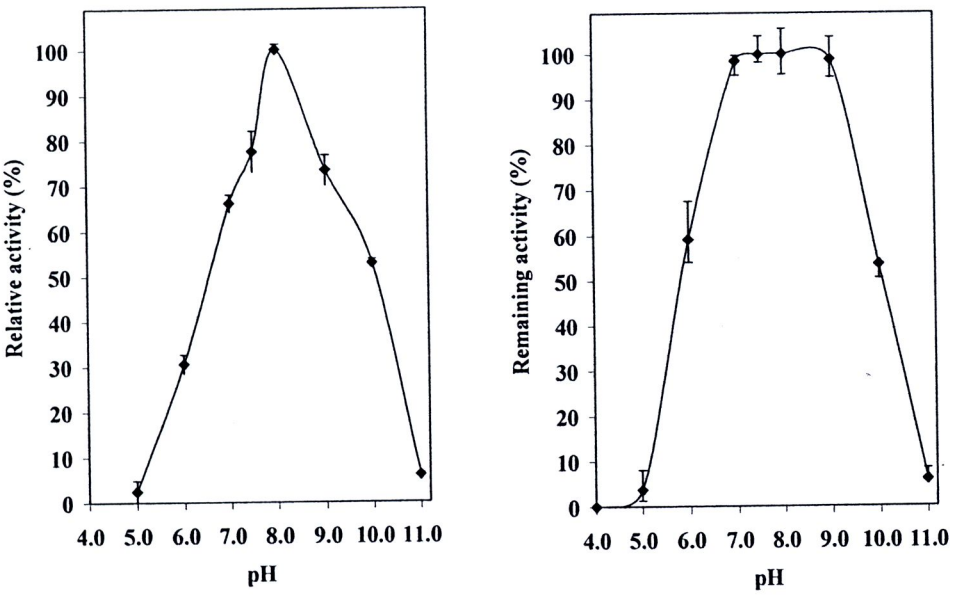

Figure 4.26 The effect of pH on protease activity (A) and stability (B) of the purified protease from ND1-1. Means of triplicate determinations are presented, and bars indicate standard deviation. Absence of bars indicates that errors are smaller than symbols. An activity (A) was analyzed using casein as a substrate at various pH (5.0–11.0) by using Britton and Robinson universal buffer. For the stability test (B), the enzyme was incubated at various pH (5.0–11.0) using Britton and Robinson universal buffer for 24 h at 4°C. The remaining activity was analyzed. Activity analyzed by using casein as substrate, pH 7.5 at 37°C for 1 h was set as 100%.

4.3.4.4 Optimal salt concentration and salt stability

Protease activity was measured in the presence of various NaCl (0-30% w/v NaCl), the maximal activity (3.2 Units) was obtained in the presence 5% w/v NaCl, while higher than 50% of the maximal activity was detected in the presence of 15% w/v NaCl or in the absence of NaCl. Protease was stable for at least 24 h when incubated in the 50 mM Tris-HCl buffer pH 8.0 containg 5-30% w/v NaCl. More than 50% of stability were lost in absence of salt. The effect of NaCl concentration on activity and stability wassimilar to another proteases moderately halophilic bacteria, but different from zinc metalloprotease and serine metalloprotease of *Salinivibrio*, sp. AF 2004 including protease of *Salinivibrio* sp 18AG^{T}. The above report protease had optimal NaCl concentration at 0-2.5% w/v and more than 50% of Activity were lost in presence of 10% w/v NaCl (Amoozegar, et al., 2007; Amoozegar, et al., 2007b and Lama et al., 2005). Many reports showed protease having maximal activity in low salt condition when casein was used as substrate but in high salt condition when synthetic substrate was used as substrate. Because in the presence of salt concentration, casein becomes hydrophobic which make it poorly soluble in water then it was unavialable as a target for proteolytic activity. The stability of ND1-1 protease in absence salt was similar to that of *Filobacillus* sp., RF2-5 protease, but incontrast with *Halobacillus* sp. SR5-3 protease which protease was completely inactivated.

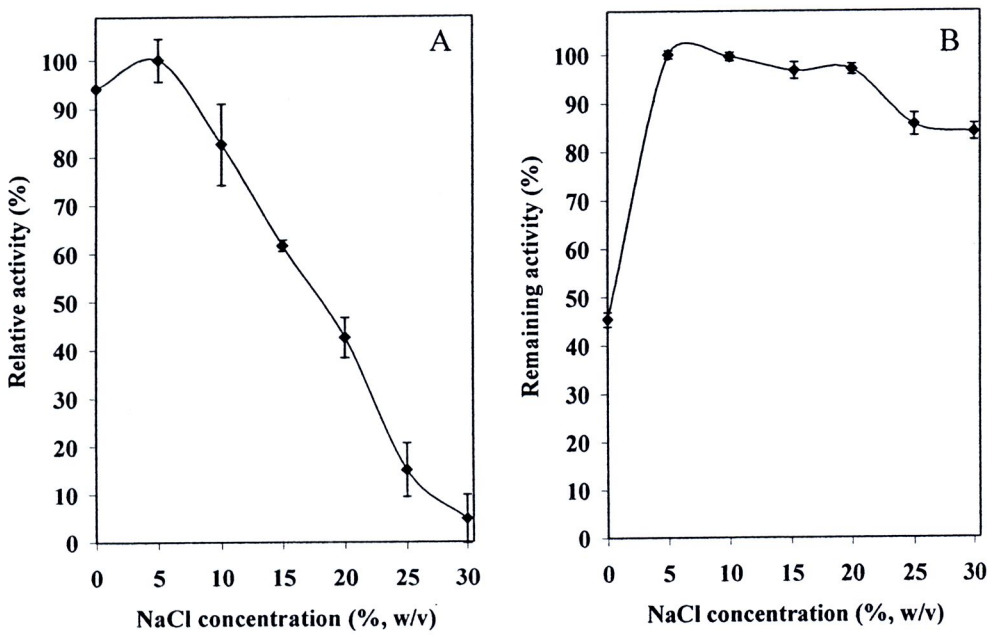

Figure 4.27 The effect of NaCl on protease activity (A) and stability (B) of the purified protease from ND1-1. Means of triplicate determinations are presented, and bars indicate standard deviation. Absence of bars indicates that errors are smaller than symbols. Activity (A) was analyzed in various NaCl concentration using casein as a substrate at 55°C, pH 8.0 for 10 min. For the stability test (B), the enzyme was incubated at 30°C in 50 mM Tris-HCl buffer (pH 7.0) containing various NaCl concentrations for 24 h, then remaining activity was analyzed. Activity analyzed by using casein as a substrate in the presence of 10% w,v NaCl, at 55°C, pH 8.0 for 10 min.

4.3.4.5 Effect of inhibitors on the purified protease activity

Activity of the purified protease of ND1-1 was strongly inhibited by metal ion chelators, EDTA which 94.6% of activity was inhibited. There was no significant effect of an inhibitors of acid proteinase, serine protease, cysteine proteinase, trypsin-like proteinase and also phopharamidon; the competitive peptide inhibitor of thermolysin-like metalloprotease (Table 4.27). The results suggested that the protease from ND1-1 was a metallo-protease and it might be related to zinc

metaloprotease protease of *Salinivibrio* sp. strain AF2004 (Amoozegar, et al., 2007). In contrast to other proteases previously purified and characterized from moderately halophilic bacteria, *Pseudoalteromonas* sp. strain CP76 (Sanchez-porro et al., 2003) and *Salinivibrio costicola* subsp. *alcaliphilus* (Lama et al., 2005), the ND1-1 protease was not were inhibited by PMSF. The ND1-1protease was unaffected by serine protease inhibitor .

Table 4.27 Effect of various protease inhibitors on activity of ND1-1 protease.

Inhibitors	Targeted enzyme	Final concentation	Inhibition (%)
Leupeptin	Trypsin-like and some cysteine proteinase	0.1mM	0
Trypsin inhibitor I	Trypsin-like proteinase	0.1g/l	0
PMSF	Serine protease	1mM	3.6
Chymostatin	Chymotrypsin-like serine proteinase	0.1mM	4.2
Pepstatin	Acid proteinase	2μm	5.3
E64	Cysteine proteinase	10μM	6.4
EDTA	Metalloproteinase	10mM	94.6
EGTA	Metalloprotease	1mM	0
Phosphoramidon	Thermolysin	10μM	5.54

4.3.4.6 Effect of metal ion on the purified protease activity

Effect of divalent metal ions on activity of the purified ND1-1 protease was examined. Ca^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+} ions enhanced an activity, while Cu^{2+} inhibited the activity (Table 4.28). Zn^{2+} more than 0.1 mM inhibited metallo-protease (Zhang et al., 2003 and Karbalaei-Heidari et al., 2006) . The inhibition was probably due to the formation of zinc monohydroxide that bridged the catalytic zinc ion to side chain of an active site (Larsen and Auld. 1991).

Table 4.28 Effect of divalent metal ions on activity of purified ND1-1 protease

Metal ion (1mM)	Relative activity (%)
Ca^{2+}	113.0
Cu^{2+}	80.7
Fe^{2+}	109.8
Mg^{2+}	119.0
Mn^{2+}	98.1
Zn^{2+}	106.0

4.5 Conclusions

The moderately halophilic bacterium, strain ND1-1 produced extracellular protease at the middle of exponential phase. The highest protease production occured at the beginning of stationary phase. The maximum protease production was achieved when strain ND1-1 was cultivated in a modified no.377 medium which casamino acids was omitted, containing 10% w/vNaCl, pH 8.0 and incubated at 37°C with shaking (200rpm) for 2 days. Under the optimal conditions, crude protease produced increased 6.25 times. The purified ND1-1 protease was monomeric protein with a molecular mass of about 36.8 kDa (calculated by gel filtration method). The enzyme had a maximal activity in the presence of 5% w/v NaCl, pH 8.0 and at 55°C. More than 50% of the activity remained when in the presence of 5-30%w/v NaCl, pH 5.0-9.0 and at 30–55°C for 1 h. The ND1-1 protease was identified to be metallo-protease without Ca^{2+} in the catalytic domain. Because of ND1-1 protease did not inhibited by EGTA (Specific Ca^{2+} chelating agent).