# CHAPTER IV

# RESULTS AND DISCUSSION

## Screening of protease-producing halophilic bacteria

### 1 Screening of protease-producing halophilic bacteria on agar plate

The halophilic bacteria were isolated from total 65 sample of Pla-ra in Thailand (Table 8). Of the strains tested, 46 exhibited protease activity in skim milk agar plate containing 10%(w/v)NaCl, but only 30 isolates showed clear zone on 15%(w/v) NaCl (Table 9). Twelve from the thirty isolates, strains A, BKN1-1, BN1-1, HUT1-1, NB2-1, NB3-1, ND1-1, PL1-1, PL3-1, PR5-1, SS1-1, and TSY 4-4 were selected for the protease activity assay in broth cullture. In consideration of protease production in 10% and 15%(w/v) NaCl, 5 strains were selected for further study.

### 2. Protease activity assay

To measure protease production ability more quantitatively, the culture supernatant of each strain was used for protease assay method. Coolbear *et. al.*(1991) reported that there is not necessarily a good correlation between zones of clearing around colonies on milk-agar plates and levels of protease produced.

From Figure 4, strain PR5-1 and NB2-1 produced maximal protease at 0.025 and 0.0242 units/mg protein, respectively, in the presence of 10% (w/v) NaCl. In the presence of 15% (w/v) NaCl, strain NB2-1 produced more protease (0.0152 units/mg protein) than PR5-1 ( 0.0089 units/mg protein) From this result , the strain NB2-1 was selected for further study.

Table 7. Source of isolations

| Location, Province | Sample code | No. sample |
|---|---|---|
| Nang lueng, Bangkok | BN1, BN2 | 2 |
| Thaewast, Bangkok | BT1, BT2, BT3 | 3 |
| Suanphloo, Bangkok | BS1, BS2, BS3 | 3 |
| Banna, Nakhon Nakyok | NB1, NB2, NB3, NB4, NB5, NB6 | 6 |
| Chiangkhan, Loei | L1 | 1 |
| Nakhon Ratchasima | RM1, RM2, RM3 | 3 |
| Ta-lad Thai, Bangkok | PT1, PT2, PT3, PT4, PT5, PT6 | 6 |
| Thonglaw, Bangkok | K1, K2 | 2 |
| Kalasin | G1 | 1 |
| Yangta-lad, Kalasin | A | 1 |
| Chaiyaphum | CH1 | 1 |
| Samrong, Samut Prakarn | BSR1, BSR2, BSR3, BSR4 | 4 |
| Bangkhunsri, Bangkok | BKS1, BKS2, BKS3 | 3 |
| Bangkhunnon, Bangkok | BKN1 | 1 |
| Prachinburi | PR1, PR2, PR3, PR4, PR5, PA1, PC1, PC2 | 8 |
| Donglakhorn, Nakhon Nakyok | ND1, ND2, ND3, ND4, ND5 | 5 |
| Singburi | SS1, SS2 | 2 |
| Phrannok, Bangkok | PL1, PL2, PL3 | 3 |
| Samrong, Samutprakarn | TSR1, TSR2, TSR3, TSR4, TSR5, TSR6 | 6 |
| Samyan , Bangkok | TSY1, TSY2, PaSY1, PaSY2 | 4 |
| Dairy Hut, Singburi | HUT | 1 |
| Total | | 65 |

Table 8. Protease activity of the isolates on skim milk agar

| No. | Isolate No. | Protease activity (Clear zone) | |
|---|---|---|---|
| | | 10%NaCl | 15%NaCl |
| 1 | A | + | ++ |
| 2 | BKN1-1 | +++ | +++ |
| 3 | BKS1-1 | +++ | - |
| 4 | BN1-1 | +++ | ++ |
| 5 | BSR1-1 | +++ | + |
| 6 | BSR3-1 | +++ | + |
| 7 | BSR4-1 | +++ | + |
| 8 | BT3-1 | +++ | + |
| 9 | HUT1-1 | ++ | ++ |
| 10 | HUT1-2 | + | - |
| 11 | HUT1-3 | + | + |
| 12 | L1-1 | + | - |
| 13 | NB2-1 | ++ | +++ |
| 14 | NB3-1 | ++ | ++ |
| 15 | ND1-1 | +++ | +++ |
| 16 | ND2-1 | + | + |
| 17 | PA1-1 | + | + |
| 18 | PaSY1-1 | +++ | + |
| 19 | PaSY1-2 | +++ | + |
| 20 | PaSY1-3 | + | - |
| 21 | PaSY2-1 | + | - |
| 22 | PaSY2-2 | + | - |
| 23 | PL1-1 | +++ | +++ |
| 24 | PL3-1 | +++ | +++ |
| 25 | PR2-1 | ++ | + |

Table 8. (Cont) Protease activity of the isolates on skim milk agar

| No. | Isolate No. | Protease activity (Clear zone) | |
|---|---|---|---|
| | | 10%NaCl | 15%NaCl |
| 26 | PR2-2 | +++ | + |
| 27 | PR5-1 | ++ | +++ |
| 28 | PR5-2 | + | + |
| 29 | PT2-1 | ++ | + |
| 30 | PT4-1 | ++ | + |
| 31 | PT5-1 | + | + |
| 32 | PT6-1 | ++ | + |
| 33 | RM1-1 | + | + |
| 34 | RM2-1 | + | + |
| 35 | RM3-1 | + | - |
| 36 | SS1-1 | +++ | ++ |
| 37 | TSR1-1 | + | - |
| 38 | TSR2-1 | + | - |
| 39 | TSR2-2 | + | - |
| 41 | TSR6-1 | + | - |
| 42 | TSY1-2 | ++ | - |
| 43 | TSY2-1 | + | - |
| 44 | TSY2-3 | ++ | - |
| 45 | TSY4-2 | + | - |
| 46 | TSY4-4 | +++ | +++ |

+++strong; ++ moderate+ weak; - negative

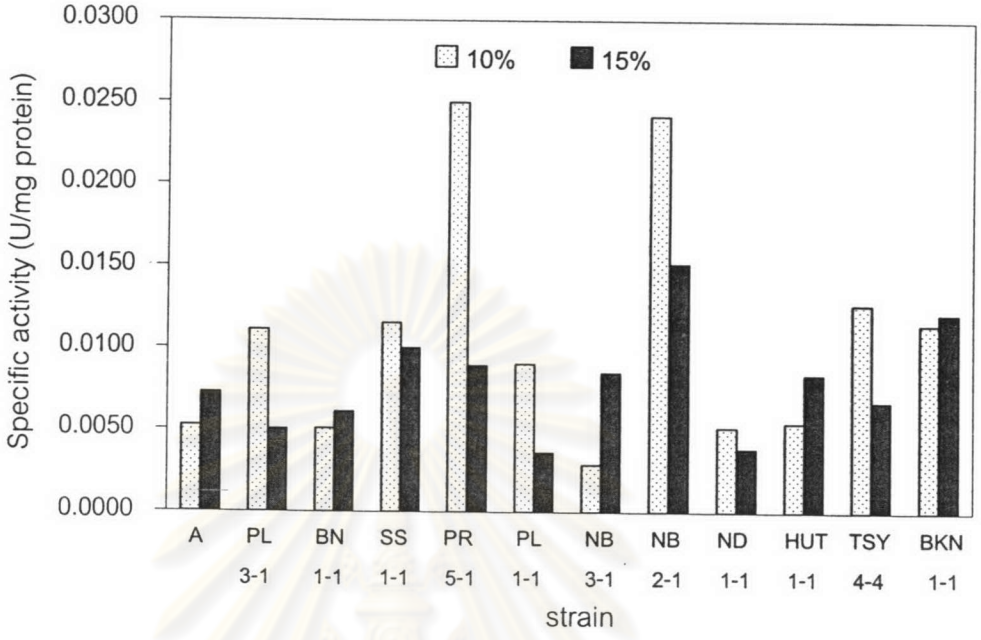


Figure 4. Protease activity of the 12 strains tested.

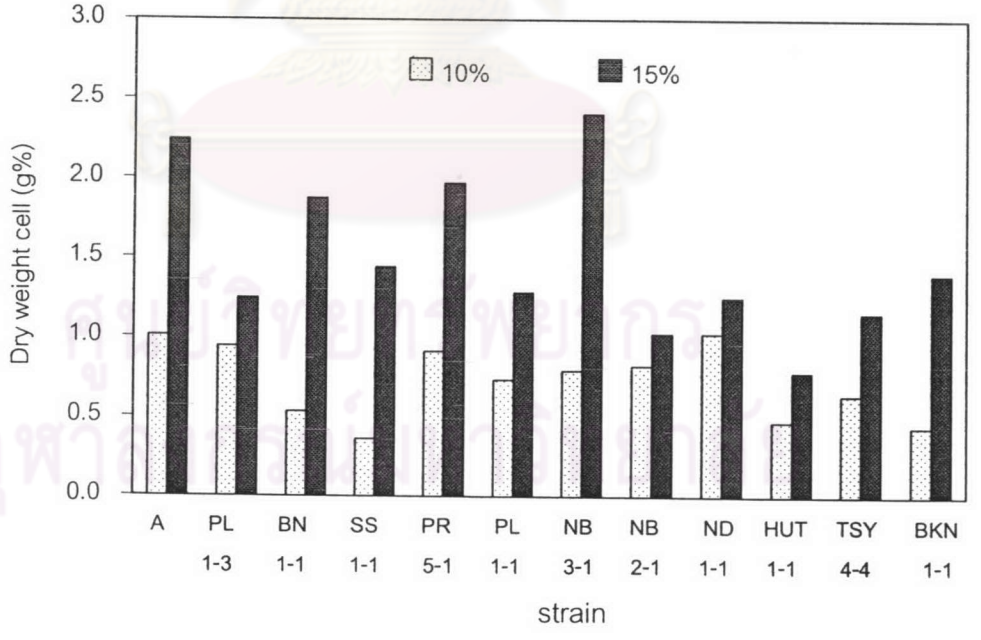


Figure 5. Dry cell weight of the 12 strains tested when grown in halobacterium JCM No. 168 containing 10% or 15% (w/v) NaCl.

Identification of strains

1. Cell morphology and cultural characteristics

All the isolaltes were rod-shaped, aerobic, Gram-positive bacteria. Some were Gram-variable upon the culture aged. Colonies were cream white or cream with pale yellow. They produced catalase, oxidase, and DNase, and grew in the medium containing 10-15% NaCl, at pH 6, and at pH 7.2. Most of them grew at 45 and 50 °C. Casein and gelatin were positive. Urease and indole test were negative. Nitrate reduction, hydrolysis of L-arginine, L-tyrosine, starch and Tween 80 were variable characteristics as shown in Table 9. Acid was produced from glucose but not from melezitose and most of the isolates produced acid from D-cellobiose, D-fructose, glycerol, *myo*-Inositol, maltose, and D-ribose (Table10). All the isolates were moderately halophilic bacteria based on their weak growth in the absence of NaCl (Heyndrickx et. al., 1998 ).

The selected strain, NB2-1 was rods, approximately 0.6-0.8x.1.5-3.5 µm. The strain motiled by means of peritrichous flagella. Ellipsoidal endospores of BN1-1 and NB2-1 were presented at the terminal position (Figures 6 and 7). Colonies of strain NB2-1 was circular to slightly irregular, raised, translucent, creamy white agar plate.

Strain NB2-1 could grow at 30 - 50 °C but not at above 50 °C and grew optimally at pH 6-9 but did not grow at pH 5. This strain grew in presence of 0 – 20%NaCl but not grow in the presence of more than 20% NaCl. The strain NB2-1 did not grow under anaerobic conditions. Casein, gelatin, Tween 80 and aesculin were hydrolysed, except the L-tyrosine and starch. The phenotypic properties of strain NB2-1 were shown in Tables 9-10.

2. Chemotaxonomic characteristics and DNA base composition

Eleven strains contained *meso*-diaminopimelic acid as the diagnostic diamino in the cell wall peptidoglycan, except strain ND1-1. The predominant menaquinone was menaquinone with seven isoprene units (MK-7) for 5 tested strains . The genomic DNA G+C content ranged from 36.2 to 49.6 mol% (Table 12).

3. Phylogenetic analysis

The 16S rDNA sequence of strains BN1-1, SS1-1, and PR5-1, comprised of 1350 were analysed as the representative strains. The 16S rDNA sequence similarity of BN1-1 and the type strain *Virgibcillus marimortui* DSM12325$^T$ was 99.8% while SS1-1 and PR5-1 with the type strain *Virgibcillus halodenitrificans* DSM 10037$^T$ (=JCM 12304) was 99.7%. A phylogenetic tree of BN1-1, SS1-1, and PR5-1, generated using the neighbour-joining based on 16S rDNA sequence was showed in Figure 8.

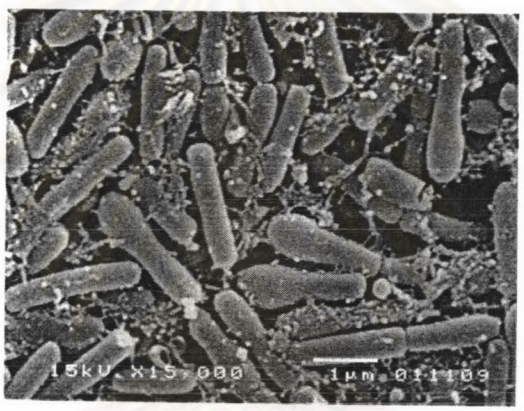

Figure. 6 Scanning electron micrograph of strain BN1-1 grown on halobacterium agar medium JCM No. 168 containing 10% NaCl at 37 ºC for 5 days.

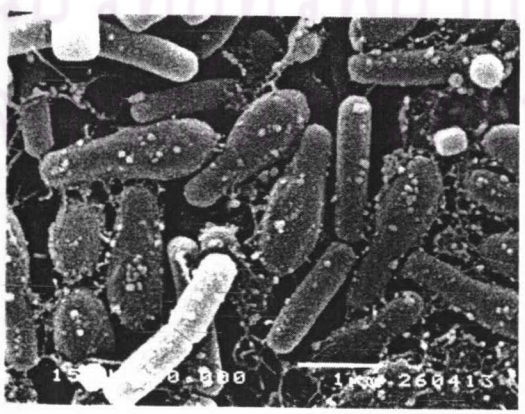

Figure. 7 Scanning electron micrograph of strain NB2-1 grown on halobacterium JCM No. 168 medium containing 10% NaCl at 37 ºC for 5 days.

Table 9. Morphological, and physiological characteristics

| No. | Isolate No. | Cell | | Growth in ( %NaCl) | | | | | Growth at pH | | | | Growth at °C | | | Catalase | Oxidase | DNAase | Urease | Indole production | L-arginine hydrolysis | Nitrate reduction | Hydrolysis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Shape | Gram | 0 | 5 | 10 | 15 | 20 | 5 | 6 | 8 | 9 | 45 | 50 | 55 | | | | | | | | Casein | Gelatin | L-tyrosine | Starch | Tween 80 |
| 1 | A | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 2 | BKN1-1 | Rods | + | + | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | - | + | + | + | - | - | + |
| 3 | BKS1-1 | Rods | + | + | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | - |
| 4 | BN1-1 | Rods | + | + | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 5 | BSR1-1 | Rods | + | - | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | - | - | + | + | - | - | - |
| 6 | BSR3-1 | Rods | + | + | + | + | + | _ | - | + | + | | - | _ | - | + | + | + | - | - | - | - | + | + | - | - | - |
| 7 | BSR4-1 | Rods | + | - | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 8 | BT3-1 | Rods | + | + | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 9 | HUT1-1 | Rods | + | - | + | + | + | - | + | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | + | + |
| 10 | HUT1-2 | Rods | + | + | + | + | + | - | - | - | + | - | + | + | - | + | + | + | - | - | + | + | + | + | - | + | - |
| 11 | HUT1-3 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | ND | + | + | + | - | - | - |
| 12 | L1-1 | Rods | + | + | + | + | + | - | - | + | + | + | - | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 13 | NB2-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | + |
| 14 | NB3-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | ± | + | + | + | - | - | - |
| 15 | ND1-1 | Rods | + | + | + | + | + | - | - | + | + | + | - | + | - | + | + | + | - | - | - | - | + | + | - | - | - |
| 16 | ND2-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | + | + | + | + | + | - | - |

Table 9. (Cont) Morphological and physiological characteristics

| No. | Isolates No. | Cell | | Growth in ( %NaCl) | | | | | Growth at pH | | | | Growth at °C | | | Catalase | Oxidase | DNAase | Urease | Indole production | L-arginine | Nitrate reduction | Hydrolysis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Shape | Gram | 0 | 5 | 10 | 15 | 20 | 5 | 6 | 8 | 9 | 45 | 50 | 55 | | | | | | | | Casein | Gelatin | Starch | L-tyrosine | Tween 80 |
| 17 | PA1-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 18 | PaSY1-1 | Rods | + | + | - | + | + | - | + | + | - | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | - |
| 19 | PaSY1-2 | Rods | + | - | + | + | + | - | + | + | - | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | - |
| 20 | PaSY1-3 | Rods | + | - | + | + | + | - | + | + | + | - | + | + | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 21 | PaSY2-1 | Rods | + | + | + | + | + | - | - | + | - | - | + | + | - | + | + | + | - | - | + | + | + | - | - | - | + |
| 22 | PaSY2-2 | Rods | + | - | - | + | + | - | + | + | - | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | + |
| 23 | PL1-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | + |
| 24 | PL3-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | - | + | + | - | - | + |
| 25 | PR2-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | - | - | + | + | + | - | - | - | + | + | + | - | + | - |
| 26 | PR2-2 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 27 | PR5-1 | Rods | + | + | + | + | + | - | - | + | + | - | + | W | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 28 | PR5-2 | Rods | + | - | + | + | + | - | - | + | + | - | + | W | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 29 | PT2-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 30 | PT4-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 31 | PT5-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | - | - | + | + | + | - | - | + | + | + | + | - | - | - |
| 32 | PT6-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |

Table 9. (Cont) Morphological and physiological characteristics

| No. | Isolates No. | Cell | | Growth in ( %NaCl) | | | | | Growth at pH | | | | Growth at °C | | | Catalase | Oxidase | DNAase | Urease | Indole production | L-arginine | Nitrate reduction | Hydrolysis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Shape | Gram | 0 | 5 | 10 | 15 | 20 | 5 | 6 | 8 | 9 | 45 | 50 | 55 | | | | | | | | Casein | Gelatin | Starch | L-tyrosine | Tween 80 |
| 33 | RM1-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | – | - | + | + | + | + | - | - | + |
| 34 | RM2-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | + | - | + | + | - | - | + |
| 35 | RM3-1 | Rods | + | + | + | + | + | - | + | + | + | + | + | + | - | + | + | - | - | - | - | + | + | + | + | - | + |
| 36 | SS1-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 37 | TSR1-1 | Rods | + | + | + | + | + | - | + | + | - | - | + | - | - | + | + | + | - | - | + | + | + | + | + | - | + |
| 38 | TSR2-1 | Rods | + | + | + | + | + | - | - | + | + | - | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 39 | TSR2-2 | Rods | + | + | + | + | + | - | + | + | - | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | - |
| 40 | TSR5-2 | Rods | + | - | + | + | + | - | - | + | + | + | + | - | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 41 | TSR6-1 | Rods | + | + | + | + | + | - | + | + | - | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | + |
| 42 | TSY1-2 | Rods | + | + | + | + | + | - | - | + | - | - | + | + | - | + | + | + | - | - | + | + | + | + | + | - | - |
| 43 | TSY2-1 | Rods | + | + | + | + | + | - | - | + | + | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 44 | TSY2-3 | Rods | + | + | + | + | + | + | - | + | + | + | - | + | - | + | + | + | - | - | + | + | + | + | - | - | + |
| 45 | TSY4-2 | Rods | + | + | + | + | + | - | + | + | + | - | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |
| 46 | TSY4-4 | Rods | + | + | + | + | + | + | - | + | - | + | + | + | - | + | + | + | - | - | - | + | + | + | - | - | - |

Table 10. Acid from carbohydrates

| No. | Isolates No. | L-Arabinose | D-Cellobiose | D-Fructose | D-Galactose | D-Glucose | Glycerol | Inulin | Myo-Inositol | Lactose | Maltose | D-Mannitol | Mannose | Melibiose | Melezitose | Raffinose | Rhamnose | D-Ribose | Salicin | Sucrose | D-Sorbitol | D-Trehalose | D-Xylose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A | - | + | + | + | + | + | - | + | - | + | - | + | - | - | - | - | + | + | ± | - | ± | - |
| 2 | BKN1-1 | - | - | + | + | + | + | - | - | - | + | - | - | - | - | - | - | + | - | + | - | - | - |
| 3 | BKS1-1 | - | + | + | - | + | + | - | - | ± | + | - | + | - | - | - | - | - | + | + | - | + | - |
| 4 | BN1-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 5 | BSR1-1 | - | - | + | + | + | + | - | - | - | - | - | ± | - | - | - | - | - | - | ± | - | - | - |
| 6 | BSR3-1 | - | - | + | - | + | + | - | - | - | + | ± | - | - | - | - | - | + | - | + | - | - | - |
| 7 | BSR4-1 | - | + | + | - | + | + | - | + | + | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 8 | BT3-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 9 | HUT1-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | + | - |
| 10 | HUT1-2 | + | + | + | - | - | + | + | + | - | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 11 | HUT1-3 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 12 | L1-1 | - | - | - | + | + | + | - | - | - | + | + | - | - | - | - | - | + | - | + | - | + | - |
| 13 | NB2-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 14 | NB3-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 15 | ND1-1 | - | - | + | - | + | ± | - | - | - | + | + | + | - | - | - | - | + | - | + | - | + | - |
| 16 | ND2-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 17 | PA1-1 | - | + | + | + | + | + | - | + | - | + | - | - | - | - | - | - | + | + | - | - | - | - |

Table 10. (Cont) Acid from carbohydrates

| No. | Isolates No. | L-Arabinose | D-Cellobiose | D-Fructose | D-Galactose | D-Glucose | Glycerol | Inulin | Myo-Inositol | Lactose | Maltose | D-Manitol | Mannose | Melibiose | Melezitose | Raffinose | Rhamnose | D-Ribose | Salicin | Sucrose | D-Sorbitol | D-Trehalose | D-Xylose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18 | PaSY1-1 | + | + | + | - | + | - | - | + | - | + | + | + | - | - | - | - | + | + | + | + | + | + |
| 19 | PaSY1-2 | - | - | + | - | + | + | - | - | - | - | + | - | - | - | - | - | + | + | - | + | - | - |
| 20 | PaSY1-3 | + | + | + | + | + | + | - | - | - | - | - | + | - | - | ± | - | + | + | + | - | + | - |
| 21 | PaSY2-1 | + | - | + | + | + | - | - | - | - | + | + | + | + | - | - | + | + | + | + | + | + | - |
| 22 | PaSY2-2 | + | + | + | - | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + | + | + | - |
| 23 | PL1-1 | - | - | + | + | + | + | - | - | - | + | - | - | - | - | - | - | + | - | + | - | + | - |
| 24 | PL3-1 | - | - | + | - | + | + | - | - | - | + | - | - | - | - | - | - | - | - | + | - | - | - |
| 25 | PR2-1 | - | + | + | - | + | + | - | + | ± | + | - | + | - | - | - | - | - | + | - | - | ± | - |
| 26 | PR2-2 | - | + | + | - | + | + | - | + | + | + | - | + | - | - | - | - | - | + | - | - | - | - |
| 27 | PR5-1 | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| 28 | PR5-2 | - | - | + | + | + | + | - | - | - | + | - | - | - | - | - | - | + | - | - | - | - | - |
| 29 | PT2-1 | - | + | + | + | + | + | - | + | - | + | - | - | - | - | - | - | - | + | - | - | - | - |
| 30 | PT4-1 | - | + | + | + | + | + | - | + | - | + | - | + | - | - | - | - | - | + | - | - | - | - |
| 31 | PT5-1 | - | - | + | + | + | - | - | - | - | + | + | + | - | - | - | - | + | - | + | - | + | - |
| 32 | PT6-1 | - | + | + | + | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 33 | RM1-1 | - | + | + | - | + | + | - | + | - | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 34 | RM2-1 | - | + | + | - | + | + | - | - | ± | + | - | + | - | - | - | - | + | + | - | - | - | - |
| 35 | RM3-1 | + | + | + | - | + | + | - | + | - | ± | + | + | - | - | + | - | + | + | + | + | + | + |
| 36 | SS1-1 | - | - | + | + | + | + | - | - | - | + | - | - | - | - | - | - | + | - | + | - | + | - |

Table 10. (Cont) Acid from carbohydrates

| No. | Isolates No. | L-Arabinose | D-Cellobiose | D-Fructose | D-Galactose | D-Glucose | Glycerol | Inulin | Myo-Inositol | Lactose | Maltose | D-Mannitol | Mannose | Melibiose | Melezitose | Raffinose | Rhamnose | D-Ribose | Salicin | Sucrose | D-Sorbitol | D-Trehalose | D-Xylose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 37 | TSR1-1 | + | + | + | - | + | + | + | + | - | + | - | + | - | - | - | - | + | + | + | + | + | - |
| 38 | TSR2-1 | - | - | + | + | + | + | - | - | + | + | + | - | - | - | ± | ± | + | - | + | - | + | - |
| 39 | TSR2-2 | + | + | + | - | + | - | + | + | + | + | + | + | + | - | + | - | + | + | + | + | + | + |
| 40 | TSR5-2 | - | + | + | + | + | + | - | + | + | + | - | + | - | - | - | - | + | + | + | - | + | - |
| 41 | TSR6-1 | + | + | + | - | + | + | - | + | - | - | + | + | + | - | - | - | + | + | + | + | + | + |
| 42 | TSY1-2 | + | + | + | - | + | - | + | - | + | - | + | + | + | - | + | - | + | + | + | + | + | + |
| 43 | TSY2-1 | + | + | + | - | + | + | - | - | + | - | + | + | - | - | - | - | + | + | + | - | + | - |
| 44 | TSY2-3 | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| 45 | TSY4-2 | + | + | + | - | + | - | - | - | - | - | + | + | - | - | - | - | + | + | + | - | + | + |
| 46 | TSY4-4 | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |

+; positive ±; variable, - ; negative

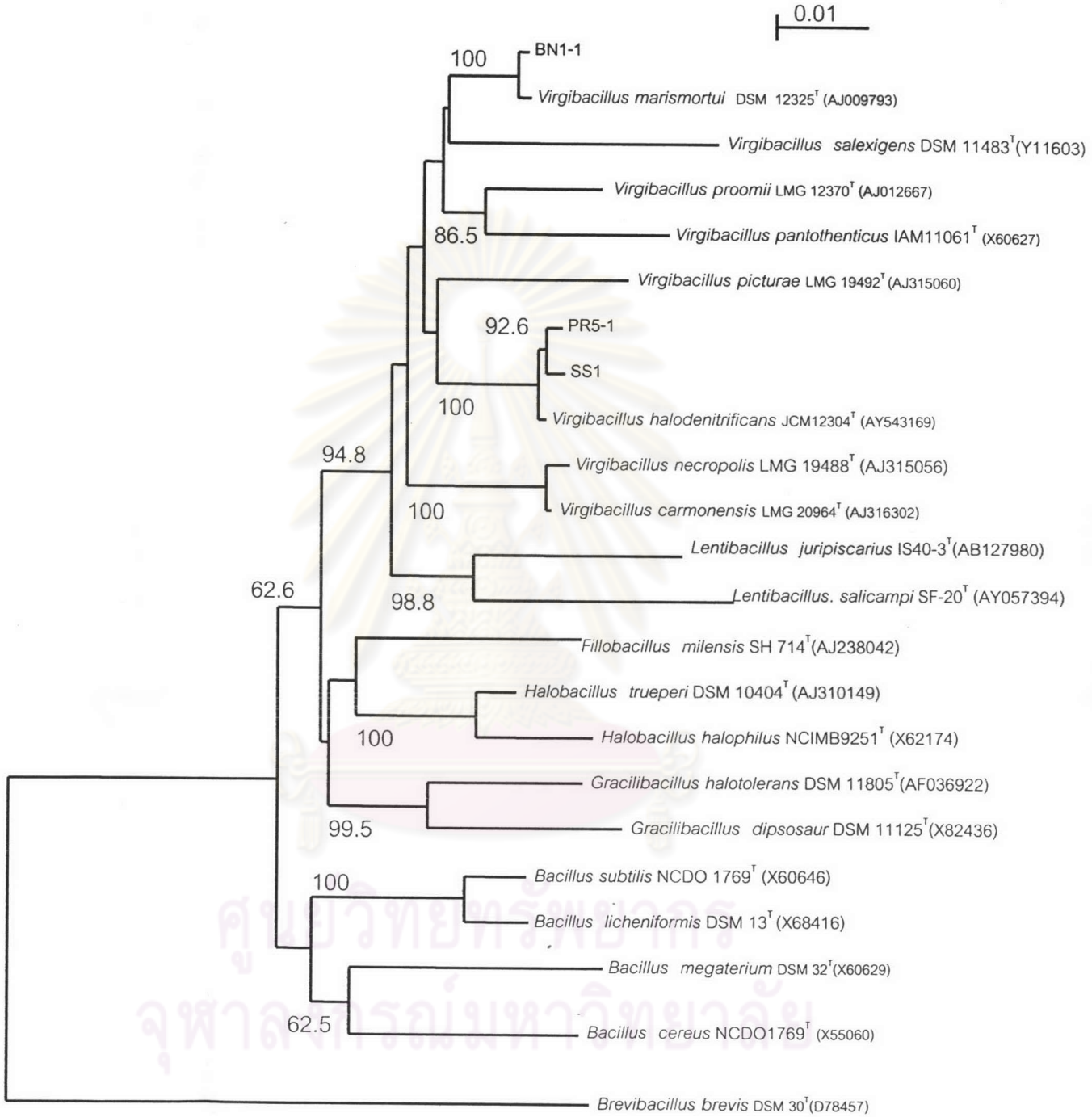


Figure. 8 Neighbour-joining-tree showing the phylogenetic position of strain BN1-1 and representatives of some other related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications .

4. DNA-DNA similarity.

Twelve strains were divided into 3 groups, 5 strains (A, BN1-1, HUT1-1, NB2-1, and NB3-1) in Group I ; 6 (BKN1-1, PL1-1, PL3-1, PR5-1, SS1-1, and TSY4-4) in Group II, and 1 (ND1-1) in Group III. Group I strains showed high degree (96.6-106.5%) of DNA-DNA similarity to the strain BN1-1(*V. marismortui* DSM12325$^T$) while the strains in Group II exhibited high degree (72.0-78.2%) of DNA-DNA similarity with the type strain of *V. halodenitrificans* JCM 12304$^T$. ND1-1 showed low DNA-DNA similarity with BN1-1 1 and *V. halodenitrificans* JCM 12304$^T$ (Table 13).

Table 11. DNA-DNA similarity of the 12 isolates and *V. halodenitrificans* JCM 12304$^T$

| Strain | %DNA similarity with labeled strains | |
|---|---|---|
| | JCM12304$^T$ | BN1-1 (*V. marismortui* DSM 12325$^T$) |
| Group I | | |
| A | 13.6 | 102.3 |
| BN1-1 | 13.9 | 100 |
| HUT1-1 | 15.9 | 101.7 |
| NB2-1 | 13.9 | 96.9 |
| NB3-1 | 11.8 | 92.7 |
| Group II | | |
| BKN1-1 | 76.9 | 13.7 |
| PL1-1 | 76.9 | 14.2 |
| PL3-1 | 77.7 | 14.29 |
| PR5-1 | 72.3 | 13.14 |
| SS1-1 | 72.6 | 13.8 |
| TSY4-4 | 71.5 | 19.2 |
| Group III | | |
| ND1-1 | 2.7 | 1.7 |
| *V. halodenitrificans* JCM12304$^T$ | 100 | 12.9 |

Table 12. Differential characteristics of the 12 isolates and *Virgibacillus* species

| Characteristics | 1 | 2 | A | BN 1-1 | HUT 1-1 | NB 2-1 | NB 3-1 | BKN 1-1 | PL 1-1 | PL 3-1 | PR 5-1 | SS 1-1 | TSY 4-4 | ND 1-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pigmentation | WC | CB | WC | WC | WC | WC | WC | CB | CB | CB | CB | CB | CB | CB |
| Spore shape | E | E | E | E | E | E | E | E | E | E | E | E | E | E |
| Spore position | T/ST | T/ST | T | T | T | T | ST | T | T | T | T | T | T | ST |
| Anaerobic growth | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| Growth on 25% NaCl | - | v(+) | - | - | - | - | - | - | - | - | - | - | - | - |
| Growth at 10 °C | - | + | w | + | w | w | w | + | w | w | w | + | + | + |
| 50 °C | + | - | + | + | + | + | + | + | + | + | w | + | + | + |
| Nitrate reduction | + | + | + | + | + | + | + | + | + | - | + | + | + | - |
| Hydrolysis of | | | | | | | | | | | | | | |
| Aesculin | + | v(+) | + | - | + | + | + | - | - | - | - | - | - | - |
| Casein | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Gelatin | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

Table 12. (Cont) Differential characteristics of the 12 isolates and *Virgibacillus* species

| Characteristics | 1 | 2 | A | BN 1-1 | HUT 1-1 | NB 2-1 | NB 3-1 | BKN 1-1 | PL 1-1 | PL 3-1 | PR 5-1 | SS 1-1 | TSY 4-4 | ND 1-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acid production from | | | | | | | | | | | | | | |
| D-Galactose | - | + | + | - | - | - | - | + | + | - | + | + | - | - |
| D-Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Fructose | + | + | + | + | + | + | + | + | + | + | - | + | + | + |
| D-Mannose | + | + | + | - | + | + | + | - | - | - | - | - | - | + |
| D-Melibiose | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| L-Rhamnose | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| D-Trehalose | - | + | ± | - | + | - | - | - | + | - | - | + | - | + |
| D-Mannitol | - | v(+) | - | - | - | - | - | - | - | - | - | - | - | + |
| *Meso* - diaminopimelic acid | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| Menaquinone | MK-7 | MK-7 | ND | MK-7 | ND | MK-7 | ND | MK-7 | ND | ND | MK-7 | MK-7 | ND | ND |
| DNA G+C (Mol %) | 40.7 | 38.0 | ND | 36.3 | ND | 38.8 | ND | ND | ND | ND | 36.2 | 38.5 | ND | 49.6 |

**Pigmentation** : WC, white creamy. CB, creamy brown. **Spore shape** : E, ellipsoidal. **Spore position** : T, terminal; S, subterminal. +, positive; w, weak positive; -, negative; v, variable **Species**: ND, not determined; 1, *Virgibacillus marismortui* 12325$^T$ ;

2, *Vergibacillus halodenitrificans* JCM12304$^T$.

Twelve strains of protease-producing halophilic bacteria were separated into 3 groups based on the phenotypic and chemotaxonomic characteristics including DNA-DNA similarity. All contained *meso*-diaminopimelic acid in the cell wall, except the strain ND1-1. The tested strains, BKN1-1, BN1-1, NB2-1, PR5-1, and SS1-1 contained MK-7 as a major menaquinone. The DNA G +C of the tested strains was 36.2 to 49.6 mol% within the same range as previous report except for the strain ND1-1 that contained 49.6 mol% (Heyndrickx et al., 1998; Arahal et al., 1999). Group I and Group II strains could be separated by some phenotypic characteristics such as the aesculin hydrolysis (Table 12). Group I strains showed high degree of DNA-DNA similarity to the strain BN1-1 while the strains in Group II exhibited high degree of DNA-DNA similarity with the type strain of *V. halodenitrificans* JCM 12304 (Table 11 ). In addition, the 16S rDNA sequence similarity of the representative strains studies were almost identical (99.7-99.8%) with *Virgibacillus marismortui* and *V. halodenitrificans* (Figure 8). Therefore, Group I strains were identified as *V. marismortui* and Group II were *V. halodenitrificans* (Stackbrandt et al., 2002; Wayne et.al., 1987; Arahal et al., 1999; Heyrman et al., 2003; Yoon et al., 2004). The highest protease-producing halophilic strain NB2-1 was identified as *V. marismortui* as mention aboved. ND1-1, Group III strain was closed to *Halobacillus* based on the cell wall and DNA base composition (Heyndrickx et al., 1998) however we left unidentified.

## Optimization of crude protease production

Optimization of crude protease production of the best protease producing Strain, NB2-1, was carried out in halobacterium medium JCM No. 168. The influence of several factors *e.g.* NaCl concentration, medium composition, initial pH, incubation temperature etc. on protease production was studied. Samples taken at different times were analyzed for protease activity in cell-free supernatant and for growth by monitoring the absorbance at 660 nm. An effective prior condition was used as the basis for the latter experiment until the optimal condition was obtained.

1 Effect of NaCl concentration and cultivation time on protease production

The strain NB2-1 was cultivated in halobacterium medium JCM No. 168 containing 0, 10, 15 or 20% (w/v) of NaCl and incubated with shaking (200 rpm) at 37°C for 5 days. The result was shown in Figure 9-10. The optimal concentration of NaCl for both protease production and growth was 15%(w/v). Maximum protease production was obtained in the medium containing 15% (w/v) NaCl after 3 days of incubation. The strain NB2-1 also able to grow in the medium without NaCl in which the highest growth was abserved at 2 days. The result indicated that the strain NB2-1 is a moderate halophilic bacterium according to the definition of Kushner (1985).

2 Effect of initial pH on protease production

The strain NB2-1 was cultivated in halobacterium medium JCM No. 168 containing 15% (w/v) NaCl which was adjusted to pH 5.0, 6.0, 7.0, 7.2, 8.0, 9.0, 10.0 or 11.0 ; and incubated at the same above condition for 3 days As shown in Figure 11, the optimal pH for protease production was 9.0. The strain NB2-1 grew at pH 5.0 to 9.0 but there was no growth and protease production at pH 10.0 and above.

3 Effect of incubation temperature on protease production

The strain NB2-1 was cultivated in halobacterium medium JCM No. 168 containing 15% (w/v) NaCl, pH 9.0 and incubated with shaking (200 rpm) at 25, 30, 37 or 45°C for 3 days. The result was shown in Figure 12 the optimal temperature for both protease production and growth was 30°C.

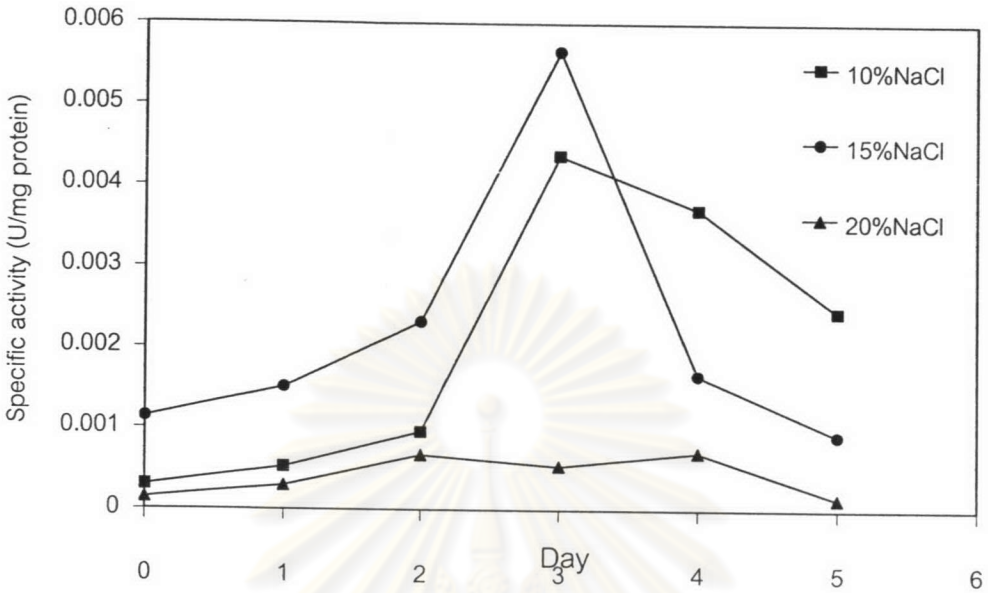


Figure 9. Effect of NaCl concentration and cultivation time on protease production of strain NB2-1.

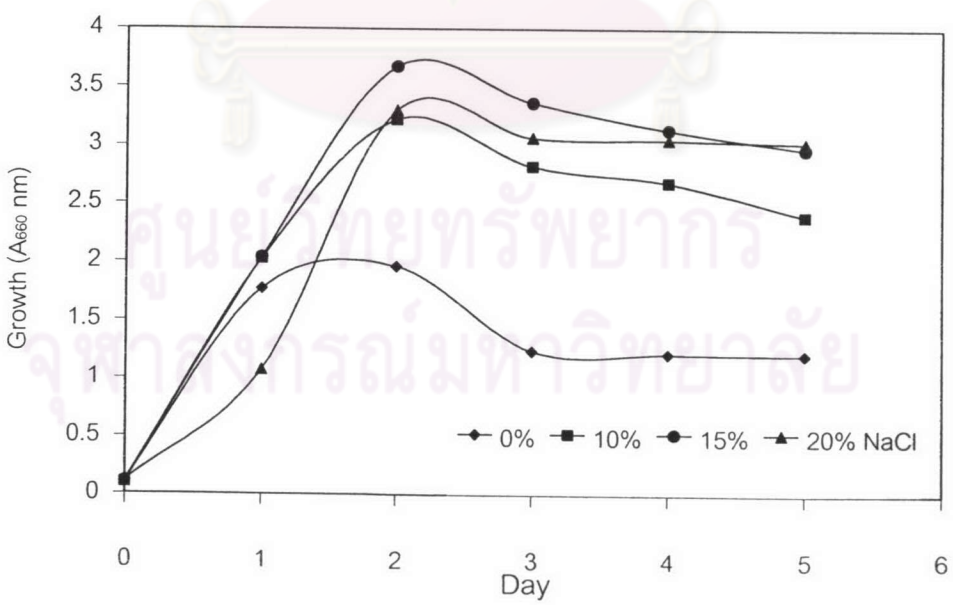


Figure 10. Effect of NaCl concentration and cultivation time on growth of strain NB2-1.

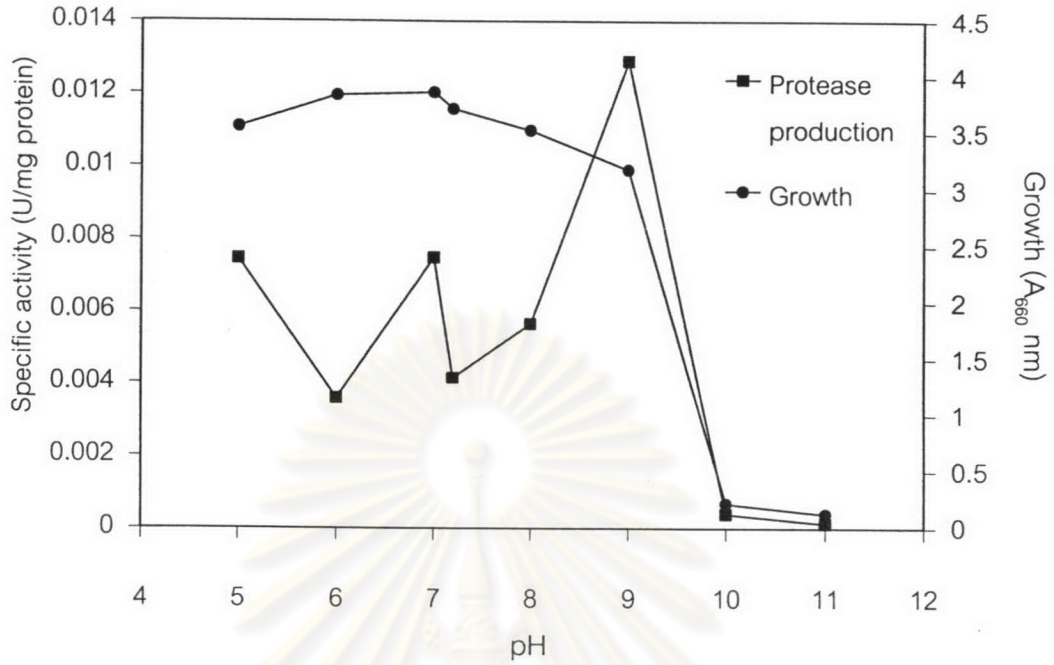


Figure 11. Effect of initial pH on protease production and growth of strain NB 2-1.

4 Effect of medium composition on protease production

The strain NB2-1 was cultivated in modified halobacterium medium JCM No. 168 containing 15% (w/v) NaCl, pH 9.0 and incubated with shaking (200 rpm) at 30°C for 3 days.The halobacterium medium JCM No. 168 was modified by eliminating of 0.5% (w/v) casamino acid or by using the following nutrients *e.g.* casein, soy flour, gelatin, skim milk or ami at 0.5%(w/v) instead of casamino acid. The result was shown in figure 13. Maximum protease production was obtained in the modified medium without casamino acid in the presence of yeast extract. Maximum growth was obtained in halobacterium medium JCM No. 168 or its modification containing soy flour instead of casamino acid at 0.5% (w/v) in the presence of yeast extract.

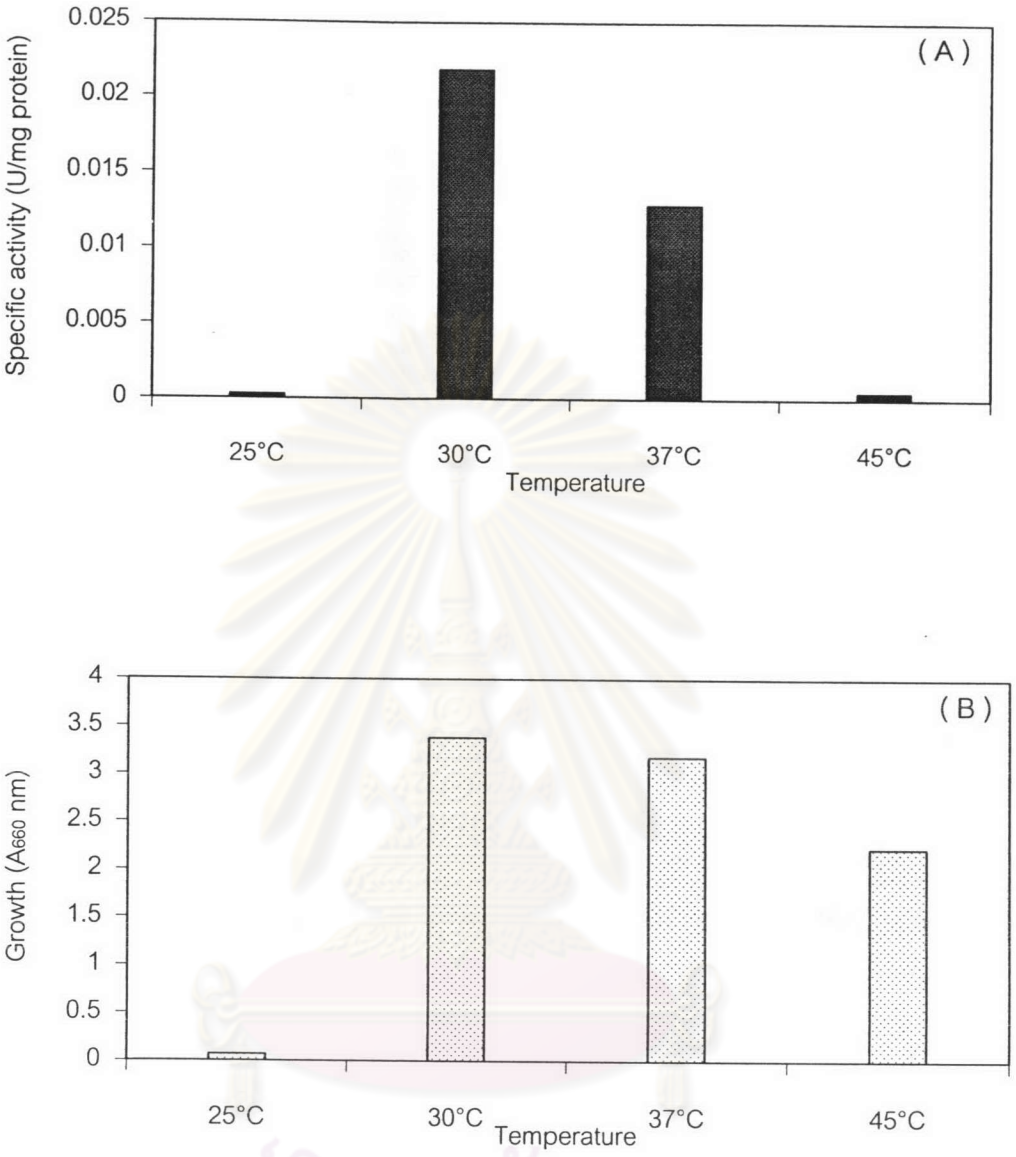


Figure 12. Effect of cultivation temperature on protease production ( A ) and growth of strain NB2-1 ( B ).

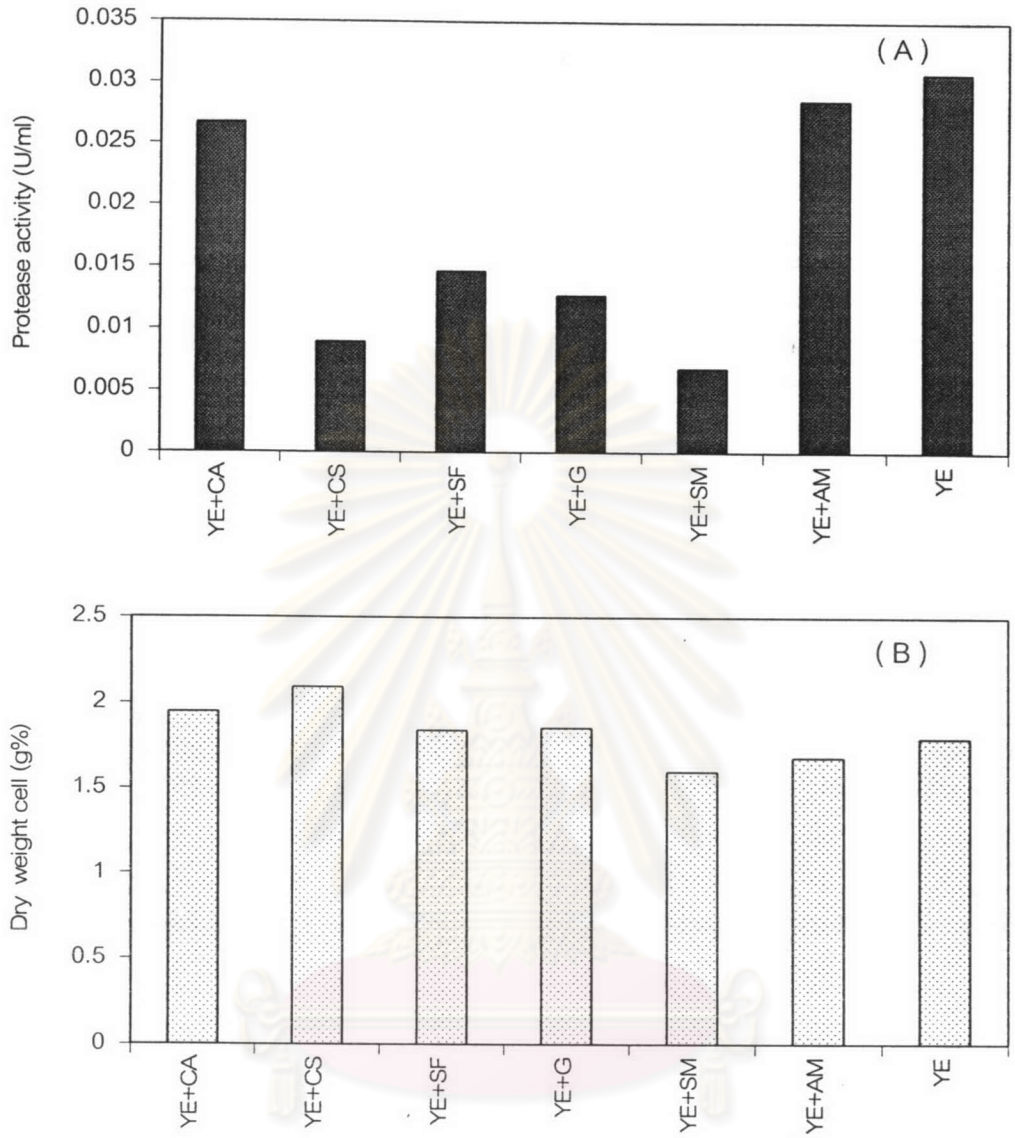


Figure 13. Effect of medium composition on protease production ( A ) and growth ( B ) of strain NB2-1.

Halobacterium medium JCM No. 168 containing 15% (w/v) NaCl,pH 9.0 was used as basal medium. The medium composition was modified by replacing of 0.5% (w/v) casamino acid with various nutrients at 0.5% (w/v) .

The symbol used: yeast extract (YE), casamino acid (CA), casein (CS), soy flour (SF), gelatin(G), skim milk (SM) and ami (AM).

To optimize the protease production, the yeast extract concentration in modified medium without casamino acid was varied. Maximal protease production and growth was obtained at 0.5% (w/v) yeast extract (Figure 14). At optimal condition, crude protease produced by strain NB2-1 increased 1.27 times.

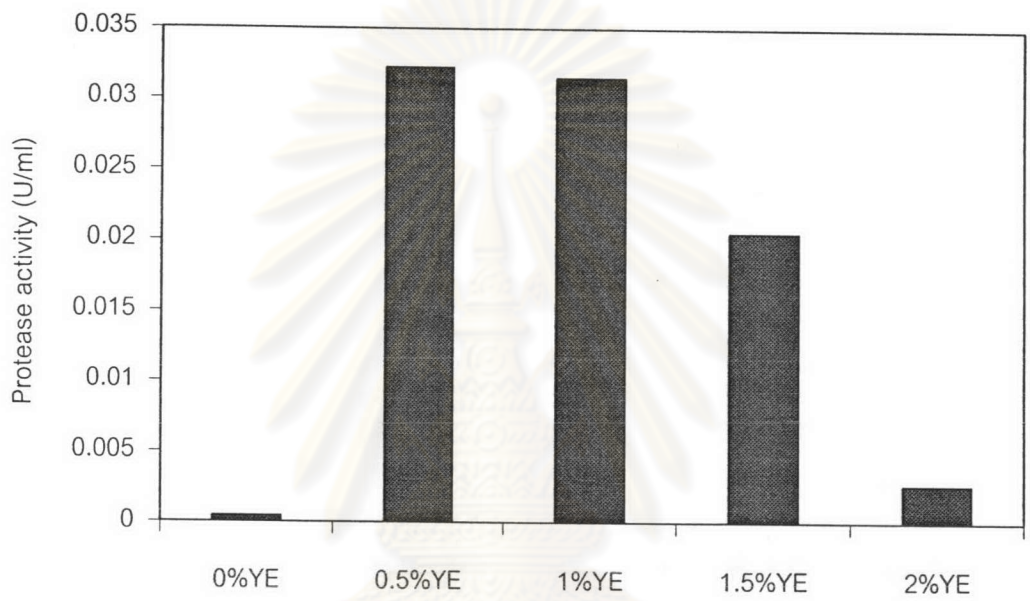


Figure 14. Effect of yeast extract concentration on protease production of strain NB2-1.
Halobacterium medium JCM No.168 without casamino acid containing 15% NaCl (w/v) NaCl, pH 9.0 was used as basal medium.



## Characterization of crude protease

Some characteristics of crude protease produced by strain NB2-1 were determined using Hammersten casein as substrate and in the presence of 10% (w/v) NaCl.

Optimal pH: Protease activity assay was carried out over the pH range of 6 to 12 at 37°C. Optimal pH for protease activity was 10.0 (Figure 15).

Optimal temperature: Protease activity at pH 10.0 was assayed at various temperature. Optimal temperature for protease activity was 50°C (Figure 14).

Optimal NaCl concentration : Protease activity was assayed at pH 10 and 50° C in the presence of various concentration of NaCl. Optimal concentration of NaCl for protease activity was 5% (w/v)(Figure 17).

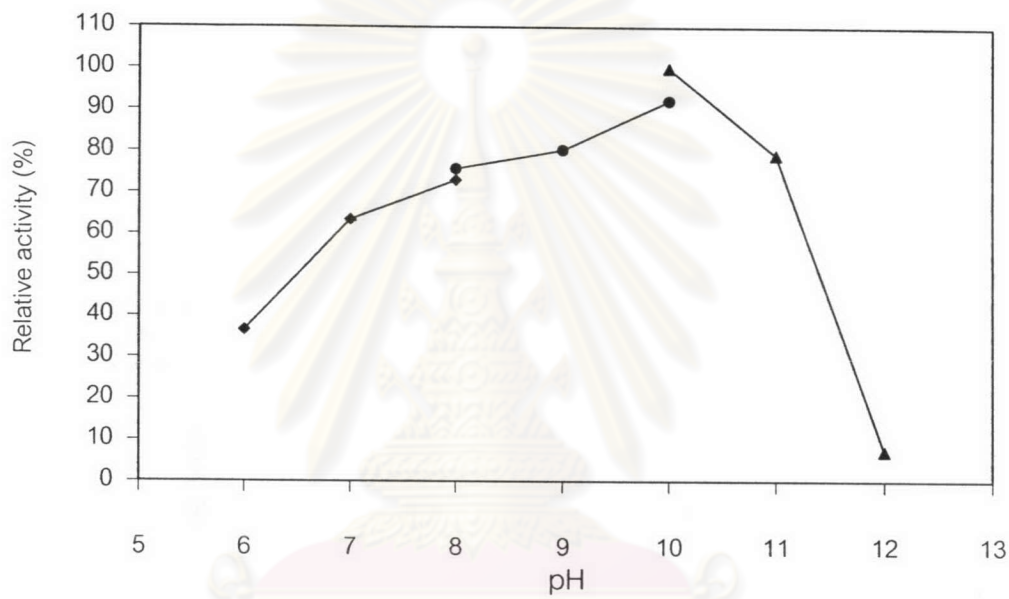


Figure 15. Effect of pH on protease activity.

Protease activity in 0.05 M $NaHCO_3$ buffer pH 10.0 was set as 100.

The buffer solution used : 0.05 M $NaH_2PO_4$ buffer (-◆-), 0.05 M $H_3BO_3$ buffer (-●-) and 0.05 M $NaHCO_3$ buffer (-▲-)

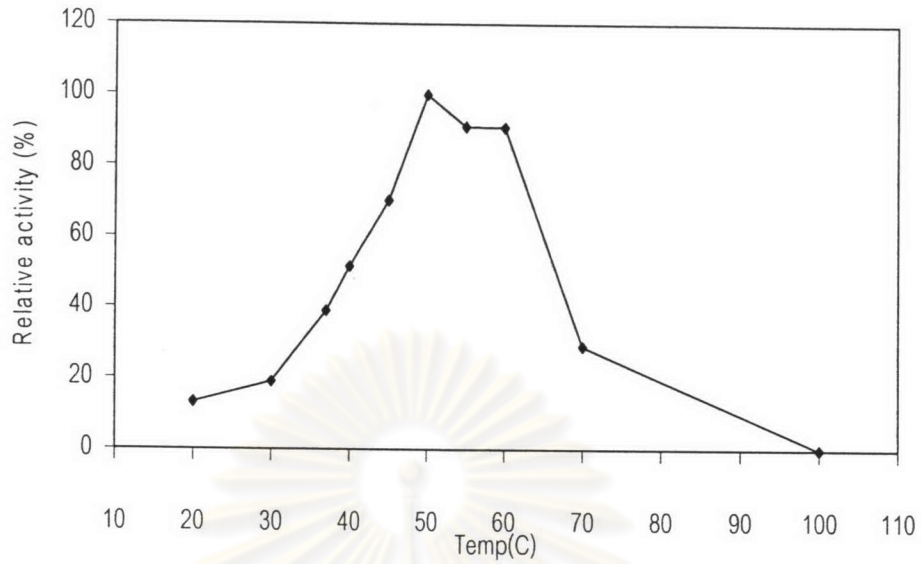


Figure 16. Effect of temperature on protease activity

Protease activity (pH 10.0) at 50° C was set as 100.

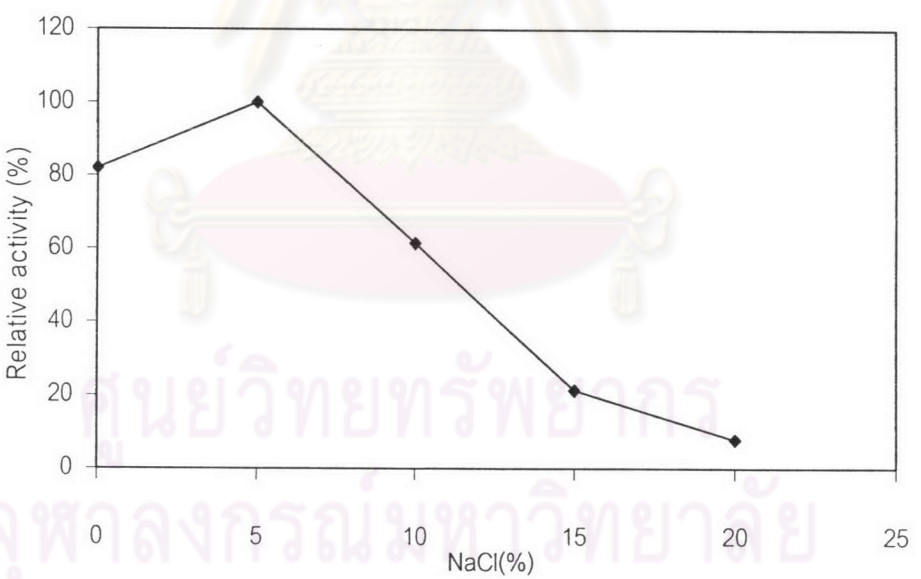


Figure 17. Effect of NaCl concentration on protease activity

Protease activity (pH 10.0 and 50° C) in the presence

Of 5% (w/v) NaCl was set as 100.

Crude protease incubated with various kind of protease inhibitors

including 20 μM E-64, 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 2 μM pepstatin and 0.2 g/l trypsin at room temperature (26-28°C) for 30 min, was assay for the remaining activity. Protease activity was measured in reaction mixture containing 10% (w/v) of NaCl concentration at pH 10.0 and 50°C for 60 min using Hammerstain casein as substrate. PMSF, trypsin and ETGA inhibited the activity at 81.68, 4.54 and 3.86% respectively (Table 13).This result suggests that the protease from strain NB2-1 is serine type protease.

Table 13. The effect of various kind of protease inhibitors on protease activity of strain NB2-1

| Inhibitors | %Inhibition |
|---|---|
| PMSF | 81.68 |
| E64 | 0 |
| EDTA | 0 |
| Pepstatin | 0 |
| Trypsin | 4.54 |
| ETGA | 3.86 |
| Control ( without inhibitor) | 0 |