CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and screening of desired bacteria

4.1.1 Screening of cellulase producing bacteria

Cellulase producing bacteria were isolated from 40 soil samples collected in Tadan district, Nakhon Nayok province; Nasuan district, Suratthani province; Hadyai district, Songkla province; Puak district, Nan province and 10 biofertilizers, by using enrichment culture method and incubating at 40 ° C. Twenty seven isolates produced clear zone surround their colonies grown on carboxymethyl cellulose-basal agar medium (Table 4.1).

Table 4.1 Sample location, date of isolation and isolate number

Location	Date of isolation	Isolate no.
Tadan, Nakhon Nayok province	September 7, 2007	T3-2, T3-3, T4-1, T6-1, T6-3, T6-4, T10-2
Nasuan, Suratthani province	May 25, 2006	PBS3, PBS4, PBS5
Hadyai, Songkla province	August 27, 2007	PBH2, PBH4
Puak, Nan province	August 27, 2007	N3-2, N12, N14-2, N16-2
Compost fertilizer	April 22, 2007	PA1-4, PA2-4, PA3-3, PA4-1, PA4-2, PA4-3, PA4-4, PF2-1, PA3-5, PD1-2, PB11
Total		**27**

Based on their cell morphological and cultural characteristics, the 27 isolates were divided into 6 groups as shown in Table 4.2. Cellulolytic activity on agar medium (clear zone diameter) and hydrolysis capacity (HC) value calculated from the clear zone diameter divided by colony diameter of each isolates grown on CMC-basal agar at 40 °C for 2 days were shown in

Table 4.3. HC value of the isolates were compared and strain PA4-1 gave maximum HC value at 10 as shown in Fig. 4.1.

Table 4.2 Cell morphological and cultural characteristics of the isolates.

Isolate no.	Colony morphology*	Cell shape	Gram	Endospore
Group1: PA4-1	Circular with raised margin, umbonate, translucent, off-white colour colonies	Rods	+	+
Group2: PBS5	Circular, umbonate, entire margins, opaque, cream colour colonies	Rods	+	+
T4-1		Rods	+	+
T6-1		Rods	+	+
Group3: T3-3	Circular, concentric, umbonate, opaque, white colour colonies	Rods	+	+
T10-2		Rods	+	+
PBS4		Rods	+	+
PA3-3		Rods	+	+
PA3-5		Rods	+	+
PA4-3		Rods	+	+
PA4-4		Rods	+	+
Group4: T3-2	Irregular and spreading, erose margins, flat, opaque, creamy colonies	Rods	+	+
T6-3		Rods	+	+
Group5: N14-2	Circular with flat and rough margins, umbonate, translucent, white cream colour colonies	Rods	-	-
PF2-1		Rods	-	-
PD1-2		Rods	-	-

Table 4.2 (Cont) Cell morphological and cultural characteristics of the isolates.

Isolate no.	Colony morphology*	Cell shape	Gram	Endospore
Group6: T6-4	Circular, convex or drop-like,	Rods	-	-
N3-2	entire margins, translucent, no	Rods	-	-
N12	pigmentation	Rods	-	-
N16-2		Rods	-	-
PA1-4		Rods	-	-
PA2-4		Rods	-	-
PA4-2		Rods	-	-
PB11		Rods	-	-
PBH2		Rods	-	-
PBH4		Rods	-	-
PBS3		Rods	-	-

+,positive; -,negative

* on PY agar medium

Table 4.3 Cellulolytic activity of the isolates on agar medium.

Isolate no.	CMC-basal agar medium		
	Colony diameter (cm)	Clear zone diameter (cm)	HC value
Group1: PA4-1	0.2	2	**10**
Group2: PBS5	0.15	0.4	2.67
T4-1	0.5	0.7	1.4
T6-1	0.4	1.55	3.88
Group3: T3-3	0.4	0.6	1.5
T10-2	0.35	0.5	1.43
PBS4	0.4	0.5	1.25
PA3-3	0.2	0.7	3.5
PA3-5	0.65	-	-
PA4-3	0.35	0.8	2.29
PA4-4	0.4	0.85	2.13
Group4: T3-2	0.9	2.1	2.33
T6-3	0.5	0.7	1.4
Group5: N14-2	0.45	0.7	1.56
PF2-1	0.2	-	-
PD1-2	0.45	0.9	2
Group6: T6-4	0.25	0.45	1.8
N3-2	0.3	0.5	1.67
N12	0.2	0.3	1.5
N16-2	0.35	0.5	1.43
PA1-4	0.3	-	-
PA2-4	0.2	-	-
PA4-2	0.2	-	-
PB11	0.25	0.9	3.6
PBH2	0.45	0.6	1.33
PBH4	0.4	0.5	1.25
PBS3	0.45	0.5	1.11

- (clear zone diameter as big as colony diameter)

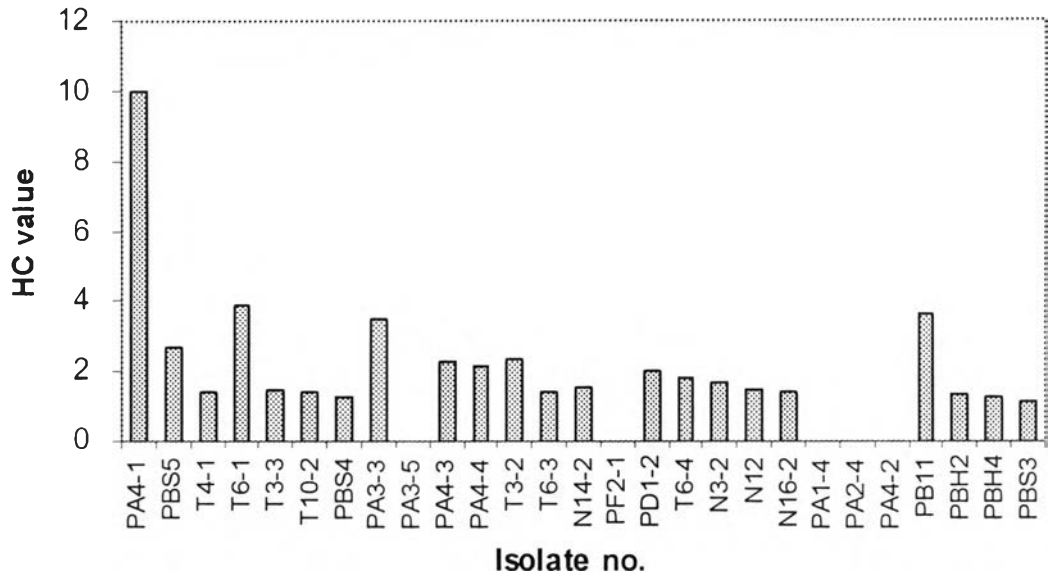

Fig. 4.1 Comparison of hydrolysis capacity (HC) value of cellulase producing bacteria isolates.

4.1.2 Quantitative cellulase producing assay

Cellulase producing bacteria isolated were grown in CMC medium at 40°c for 2 days. The supernatant obtained after centrifugation was analysed for cellulase activity. Cellulase production of the 27 isolates ranged from 0-0.015 units/ml of endoglucanase and 0-0.005 units/ml of ß-glucosidase. Two strain PB11 and PA4-1 produced endoglucanase more than 0.01 units/ml. Five strain (PA4-1, PA4-3, PA4-4, PB11, T6-1) produced ß-glucosidase more than 0.002 units/ml. Strain PB11 produced maximum endoglucanase (0.015 units/ml) and strain PA4-3 produced maximum ß-glucosidase (0.005 units/ml). Endoglucanase and ß-glucosidase production of all isolates grown in CMC medium was shown in Fig. 4.2. The HC value did not correlate to the cellulase activity (endoglucanase/ ß-glucosidase activity). This might due to an effect of synergistic action of the cellulases on clear zone production.

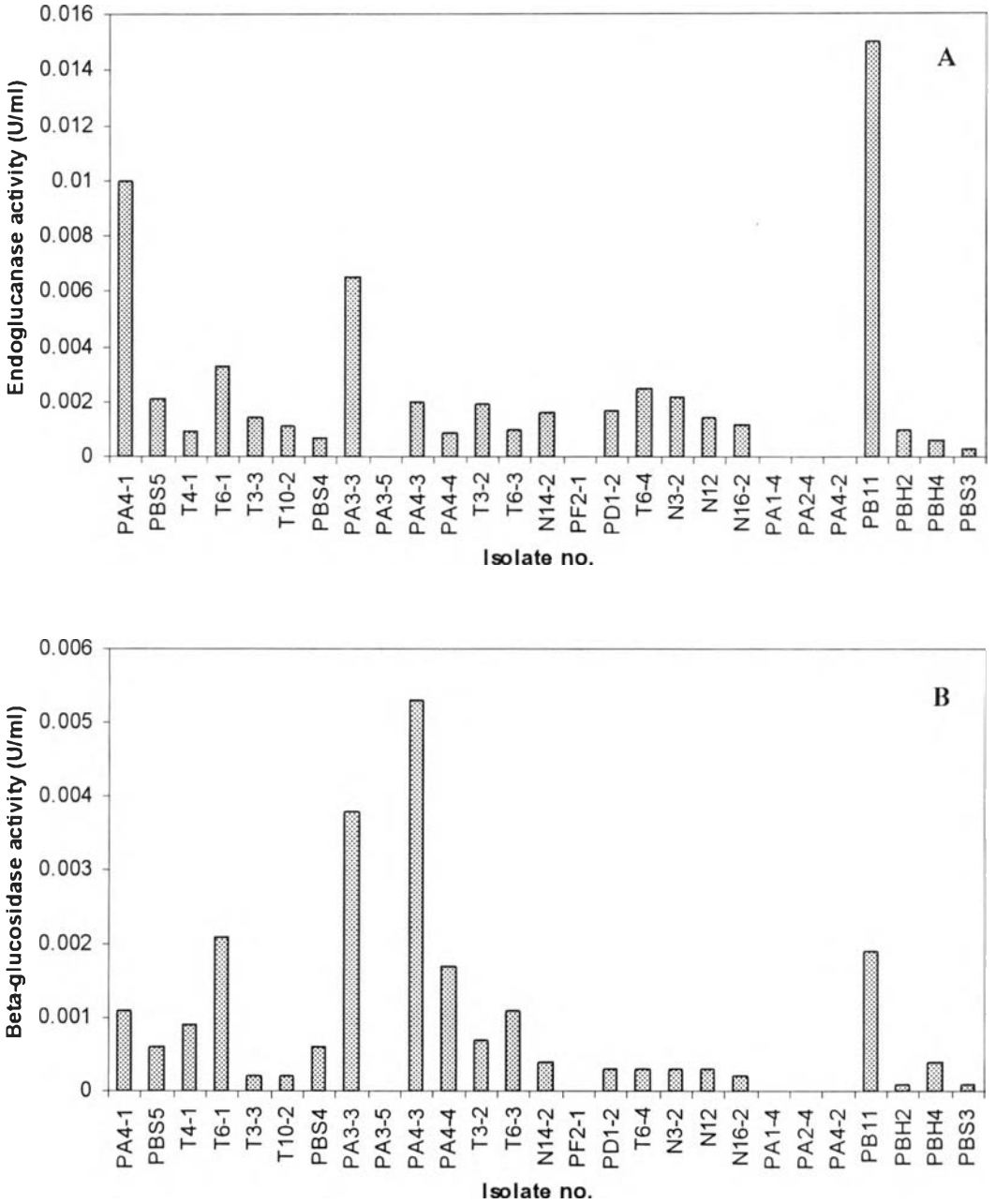

Fig. 4.2 Endoglucanase (A) and ß-glucosidase (B) production of cellulase producing bacteria isolates.

4.1.3 Screening of xylanase producing bacteria

Xylanase producing bacteria were isolated from 45 soil samples collected in Nangrong and Tadan district, Nakhon Nayok province and Puak district, Nan province by enrichment culture method and incubated at 40 ° C. Twenty four isolates produced clear zone surround their colonies grown on xylan agar medium (Table 4.4).

Table 4.4 Sample location, date of isolation and isolate number

Location	Date of isolate	Isolate no.
Nangrong, Nakhon Nayok province	April 23, 2006	PN1-2, PN8-1, PN8-2, PN8-3, PN9-3, PN12-2, PN12-3, PN13-1, PN16-5, PN20-1
Tadan, Nakhon Nayok province	May 4, 2006 September 7, 2007	PT1-1, PT1-2, PT2-3, PT2-4, PT4-2, PT6-2, PT6-3, PT8-2, TT2-2X, T3-2X, T8-1X
Puak, Nan province	August 27, 2007	N5-1X, N5-3, N9-2
Total		**24**

Based on their cell morphological and cultural characteristics, the 24 isolates were divided into 11 groups as shown in Table 4.5. Xylanase activity on agar medium (clear zone diameter) and hydrolysis capacity (HC) value calculated from the clear zone diameter divided by colony diameter of each isolates grown on xylan agar medium at 40 ° C for 2 days were shown in Table 4.6. The comparison of HC value of the bacterial isolates was shown in Fig. 4.3. Strain PN13-1 gave maximum HC value at 4.8.

Table 4.5 Cell morphological and cultural characteristics of the isolates.

Isolate no.	Colony morphology*	Cell shape	Gram	Endospore
Group1: PT4-2	Spindle with raised and entire margin, translucent, nonpigment	Rods	+	+
Group2: PN8-3	Circular with raised and entire margins, opaque, cream colour colony	Rods	+	+
Group3: PN12-2 PN12-3	Spindle, raised or low convex, entire margins, translucent, cream colour colonies	Rods Rods	+ +	+ +
Group4: PT6-2 PT6-3	Circular, umbonate, entire margins, opaque, white colour colonies	Rods Rods	+ +	+ +
Group5: PN13-1 N5-3	Circular, umbonate, entire margins, opaque, yellowish colour colonies	Rods Rods	+ +	+ +
Group6: T3-2X	Circular, umbonate, entire margin, opaque, light pink colour colony	Rods	+	+
Group7: PT2-3	Circular, umbonate, undulate margin, translucent, nonpigment	Rods	+	+
Group8: PN8-2	Irregular and spreading, rough, opaque, cream colour colony	Rods	+	+
Group9: PN1-2 TT2-2X T8-1X	Circular with lobate margins, translucent, green brown colour colony	Rods Rods Rods	- - -	- - -

Table 4.5 (Cont) Cell morphological and cultural characteristics of the isolates.

Isolate no.	Colony morphology*	Cell shape	Gram	Endospore
Group10: PN8-1	Circular, convex, entire margins,	Rods	-	-
PN9-3	opaque, cream colour colonies	Rods	-	-
PN20-1		Rods	-	-
PT1-1	Circular, convex, entire margins,	Rods	-	-
PT1-2	translucent, cream colour colonies	Rods	-	-
PT2-4		Rods	-	-
PT6-4		Rods	-	-
PN16-5		Rods	-	-
N5-1X		Rods	-	-
Group11: N9-2	Circular, entire margin, translucent, cream colour colony	Rods	-	-

+, positive; -, negative

* on PY agar medium

Table 4.6 Xylanase activity of the isolates on agar medium.

Isolate no.	Xylan agar medium		
	colony diameter (cm)	Clear zone diameter (cm)	HC value
Group1: PT4-2	0.6	1.9	3.167
Group2: PN8-3	0.4	1.75	4.375
Group3: PN12-2	0.3	1.2	4
PN12-3	0.3	1.3	4.33
Group4: PT6-2	0.5	2	4
PT6-3	0.5	1.9	3.8
Group5: PN13-1	0.5	2.4	**4.8**
N5-3	1	1.2	1.2
Group6: T3-2X	0.7	1.1	1.57
Group7: PT2-3	0.7	2.8	4
Group8: PN8-2	2.8	5	1.79
Group9: PN1-2	0.73	0.8	1.09
TT2-2X	1.5	1.7	1.13
T8-1X	0.7	1.1	1.57
Group10: PN8-1	0.37	0.4	1.09
PN9-3	0.6	0.7	1.167
PN20-1	0.55	0.6	1.09
PT1-1	0.2	0.8	4
PT1-2	0.4	0.6	1.5
PT2-4	0.35	0.6	1.71
PT6-4	0.35	0.7	2
PN16-5	0.6	0.8	1.33
N5-1X	0.8	1.2	1.5
Group11: N9-2	1	1.3	1.3

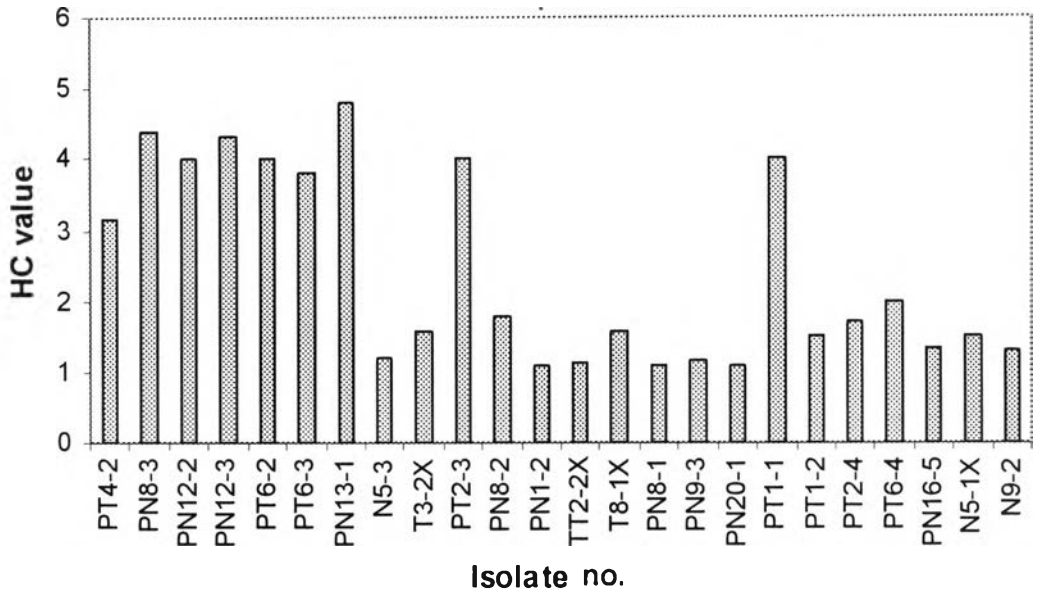

Fig. 4.3 Comparison of hydrolysis capacity (HC) value of xylanase producing bacteria isolates.

4.1.4 Quantitative xylanase producing assay

Xylanase producing bacteria isolated were grown in xylan medium at 40° C for 2 days. The supernatant obtained after centrifugation was analysed for xylanase activity. Xylanase production of the 24 isolates ranged from 0-0.48 units/ml; 3 isolates produced less than 0.01 units/ml, 12 isolates produced 0.01-0.05 units/ml, 3 isolates produced 0.06-0.07 units/ml, and 6 isolates produced 0.08-0.5 units/ml. Strain PN12-2 produced maximum xylanase at 0.5 units/ml. Xylanase production of all isolates grown in xylan medium was shown in Fig 4.4.

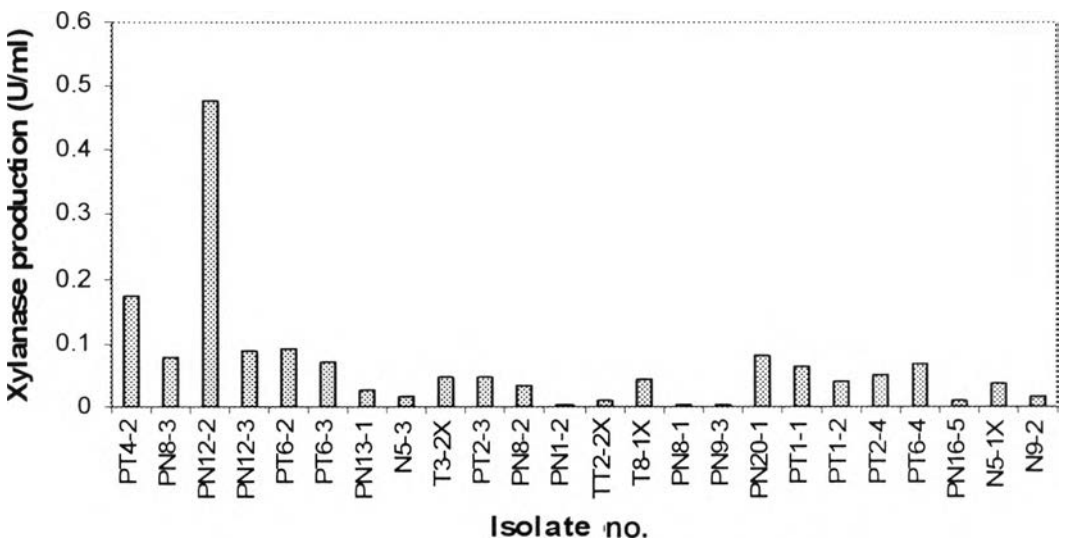

Fig. 4.4 Xylanase production of the xylanase producing bacteria isolates.

The HC value did not correlate to the xylanase activity (endoxylanase/ ß-xylosidase activity). This might due to an effect of synergistic action of the xylanase on clear zone production.

4.2 Identification

4.2.1 Identification of the cellulase producing bacteria

4.2.1.1 Cell morphological and cultural characteristics

On the basis of cell morphological and cultural characteristics of all 27 isolates as shown in Table 4.2, the isolates could be divided into 6 groups. One isolate in Group 1 was spore forming, Gram-positive rods. Colony was circular with raised margin, umbonate, translucent, off-white color. Three isolates in Group 2 were spore forming, Gram-positive bacilli. Colonies were circular, umbonate, entire margins, opaque, cream color. Seven isolates in Group 3 were spore forming, Gram-positive bacilli. Colonies were circular, concentric, umbonate, opaque, white color. Two isolates in Group 4 were spore forming, Gram-positive bacilli. Colonies were irregular and spreading, erose margins, flat, opaque, cream color. Three isolates in Group 5 were Gram-negative rods. Colonies were circular with flat and rough margins, umbonate, translucent, white cream color. Eleven isolates in Group 6 were Gram-negative rods. Colonies were circular, convex or drop-like, entire margins, translucent, no pigment. (Table 4.2)

4.2.1.2 Physiological and biochemical characteristics

Most of the isolates were catalase and oxidase positive. All isolates grew at pH 7-9. All were negative for indole production. They showed variable reaction for Methyl red, DNAase, citrate, nitrate reduction, TSI, dihydroxyacetone, gelatin hydrolysis, asculin hydrolysis, hydrolysis of L-arginine, casein, L-tyrosine, starch, and Tween 80 (Table 4.7). They showed variable acid production from carbohydrates (Table 4.8 and Appendix D). All isolates grew at 15, 20 and 45 °C, while some grew at 50 °C. The cellulase producing bacteria isolated were thermotolerant strains.

Table 4.7 Physiological and biochemical characteristics of the cellulase producing isolates.

Characteristics	Group 1 (1 isolate)	Group 2 (3 isolates)	Group 3 (7 isolates)	Group 4 (2 isolates)	Group 5 (3 isolates)	Group 6 (11 isolates)
Growth with 5% NaCl	-	+	+	+	+(2)	+(8)
Growth at pH 5.0	-	-	-(5)	-	-	+(8)
pH 6.0-8.0	+	+	+	+	+	+
pH 9.0	+	+	+	+	+	+(9)
Growth at 10 ° C	-	+	-	-	-	-
15 ° C	+	+	+	+	+(2)	+(6)
20 ° C	+	+	+	+	+	+
50 ° C	+	+	+	+	+	+(10)
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Anaerobic growth	+	+	+(4)	+	+	+
Methyl red	-	+	+(4)	+	-	-(7)
Voges-Proskauer	+	+	-(4)	-	+(2)	+(7)

Table 4.7 (Cont) Physiological and biochemical characteristics of the cellulase producing isolates.

Characteristics	Group 1 (1 isolate)	Group 2 (3 isolates)	Group 3 (7 isolates)	Group 4 (2 isolates)	Group 5 (3 isolates)	Group 6 (11 isolates)
DNase	-	-	+(4)	+	-	-(9)
Utilization of citrate	-	-	-	-	+	-(7)
TSI	N/N	K/K(1)	K/A(4)	K/A	K/K	K/A(6)
Nitrate reduction reaction	+	+(2)	+	+	+	+
Aesculin	+	+(2)	+	+	-	+(7)
Hydrolysis of						
Casein	-	+	+(5)	+	-	-(10)
Gelatin	-	-(2)	+	-	+(2)	-(6)
Starch	+	+	+(5)	+	-	-
Tween 80	-	-	-	-	-	-

+, positive; -, negative; N,neutral; K, alkaline; A, acid; Number in parentheses indicate the number of isolates showing the reaction.

Table 4.8 Acid from Carbohydrates of the cellulase producing isolates.

Acid from	Group 1 (1 isolate)	Group 2 (3 isolates)	Group 3 (7 isolates)	Group 4 (2 isolates)	Group 5 (3 isolates)	Group 6 (11 isolates)
D-Amygdalin	-	-	-(5)	-(1)	-(2)	+(8)
L-Arabinose	+	-(2)	+(5)	-	-	-
D-Cellubiose	+	+	-(6)	+	-(2)	+(6)
D-Galactose	+	-	-(4)	-	-	-(10)
Lactose	+	-(2)	+(4)	-	-	-(8)
Raffinose	-	-	+(4)	-	-	-
L-ribose	-	-	+(5)	+(1)	-	-(7)
Salicin	-	+	-(6)	+	-(2)	+(8)
D-Trehalose	-	+(2)	+(5)	+	-(2)	-(6)
D-Xylose	+	-(2)	-(4)	-	-	-(7)

+, positive; -, negative; Number in parentheses indicate the number of isolates showing the reaction.

4.2.1.3 Chemotaxonomic characteristics

The representative strains of each 6 different groups were selected and their chemotaxonomic characteristics were determined. Four tested strains, PA4-1 in Group1, PBS5 in Group 2, T3-3 in Group 3, T3-2 in Group 4 contained *meso*-diaminopimelic acid as a diagnostic diamino in the cell wall peptidoglycan (Fig. 4.5). Two tested strains, N14-2 in Group 5 and T6-4 in Group 6 did not contain *meso*-diaminopimelic acid as a diagnostic diamino in the cell wall peptidoglycan (Fig. 4.5). The predominant menaquinone (MK-7) was found in strains PA4-1 in Group1, PBS5 in Group 2, T3-3 in Group 3, T3-2 in Group 4.

On the basis of their phenotypic and chemotaxonomic characteristics, 1 isolates in Group 1 were closed to *Colnella* (Kämpfer *et al.*, 2006). Twelve isolates in Group 2, 3 and 4 showed characteristics that closed to *Bacillus* (Turnbul, 1996; Takeuchi and Hatano, 1998; Venkateswaran *et al.*, 2003) and 14 isolates in Group 5 and 6 were closed to *Pseudomonas* (Anzai *et al.*, 2000)

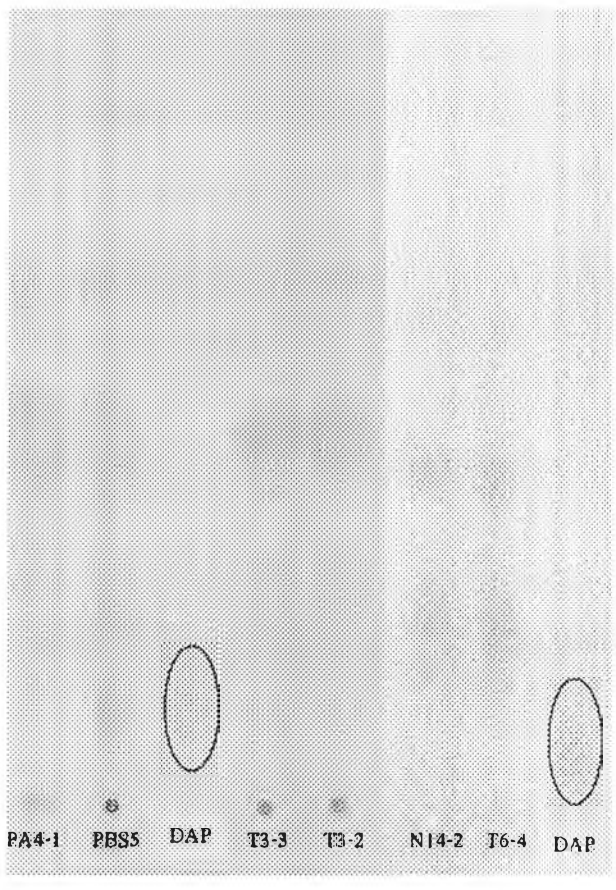

Fig. 4.5 Thin layer chromatograph showing cell wall composition of representative cellulase producing strains of each 6 different groups. (DAP: diaminopimelic acid)

4.2.1.4 16S rRNA gene sequence and phylogenetic tree analysis

The representative strain PA4-1 (1080 bp) in Group 1 showed 97.2 % similarity to *Cohnella thermotolerans* CCUG 47242^{T} (Fig. 4.6, Table 4.9). Strain PBS5 (1493 bp) in Group 2, T3-3 (1204 bp) in Group 3 and T3-2 (1167 bp) in group 4 showed 98.8, 88, and 88.7 % similarities to *Bacillus drentensis* LMG 21831^{T}, *Bacillus megaterium* IAM 13418^{T} and *Bacillus cereus* IAM 12605^{T}, respectively (Fig. 4.7, Table 4.10), Strains N14-2 (803 bp) in Group 5 and T6-4 (800 bp) in Group 6 showed 93.0 and 92.7 % similarities to *Pseudomonas pseudoalcaligenes* JCM 5968^{T} and *Pseudomonas nitroreducens* DSM 14399^{T}, respectively (Fig. 4.8, Table 4.11).

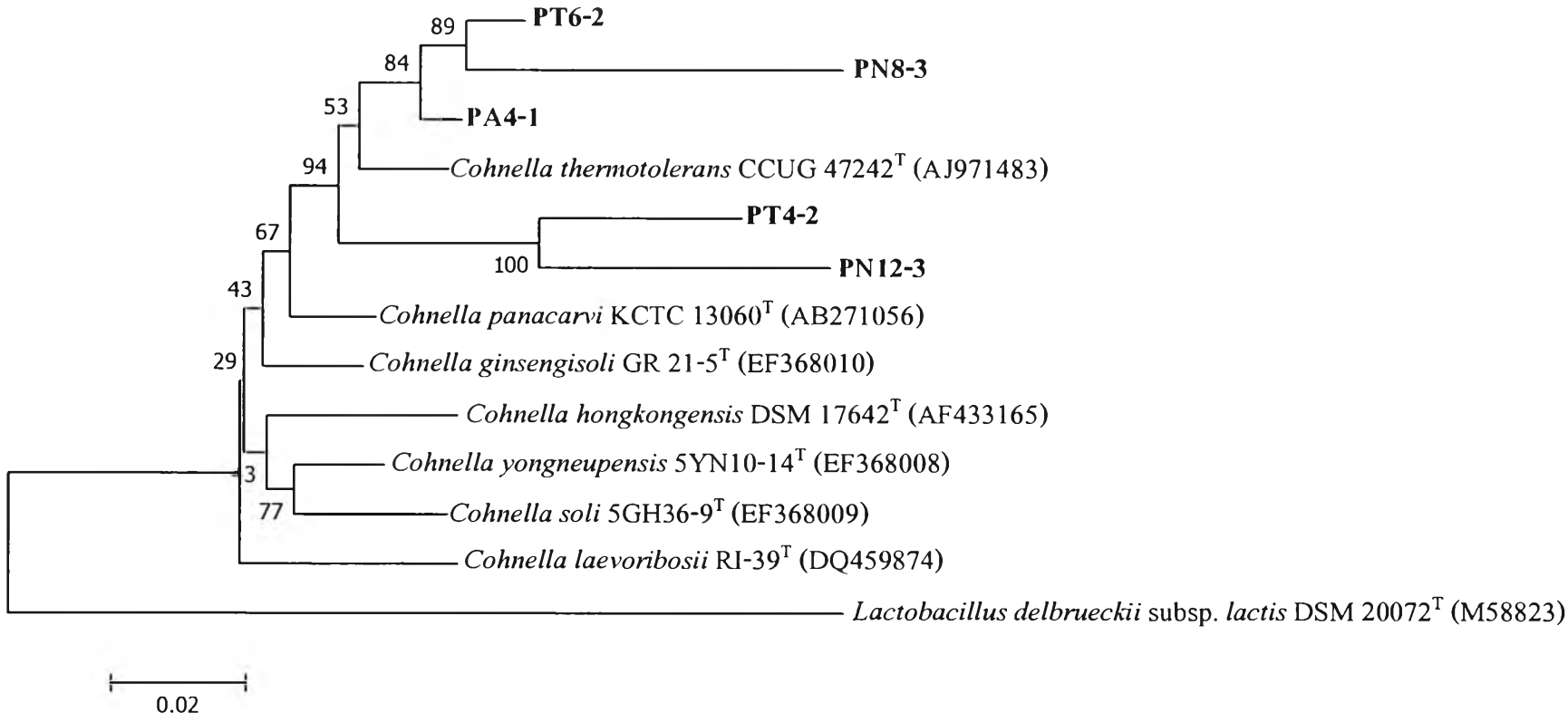

Fig. 4.6 Neighbor-joining tree showing phylogenetic position of strains PT6-2, PN8-3, PA4-1, PT4-2, PN12-3 and related taxa based on 16S rRNA gene sequences. Bar, 0.02 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications.

Table 4.9 Percentage similarities of PT4-2, PT6-2, PA4-1, PN8-3, PN12-3 and related *Colnella* species

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. PT4-2	100												
2. PT6-2	92.2	100											
3. PA4-1	91.8	98.0	100										
4. PN8-3	88.3	93.5	93.0	100									
5. PN12-3	92.7	90.3	90.4	87.8	100								
6. *C. thermotolerans* CCUG 47242^T	92.0	**96.4**	**97.2**	**91.4**	90.7	100							
7. *C. panacarvi* KCTC 13060^T	**92.4**	95.1	96.2	90.1	**91.2**	95.7	100						
8. *C. ginsengisoli* GR 21-5^T	91.5	93.9	94.9	89.0	90.4	95.8	97.0	100					
9. *C. yongneupensis* 5YN10-14^T	89.8	93.2	94.2	88.3	88.7	94.4	96.5	96.5	100				
10. *C. soli* 5GH36-9^T	89.3	92.1	93.0	87.1	88.1	93.3	95.1	95.8	96.4	100			
11. *C. laevoribosii* RI-39^T	89.4	92.7	93.9	88.5	87.6	94.2	94.8	94.9	95.1	93.5	100		
12. *C. hongkongensis* DSM 17642^T	89.3	93.6	94.5	88.7	87.9	95.0	94.6	94.7	95.3	94.7	93.5	100	
13. *L. lactis* DSM 20072^T	77.1	79.6	81.4	75.0	75.7	81.0	82.0	82.4	81.3	81.1	81.0	81.4	100

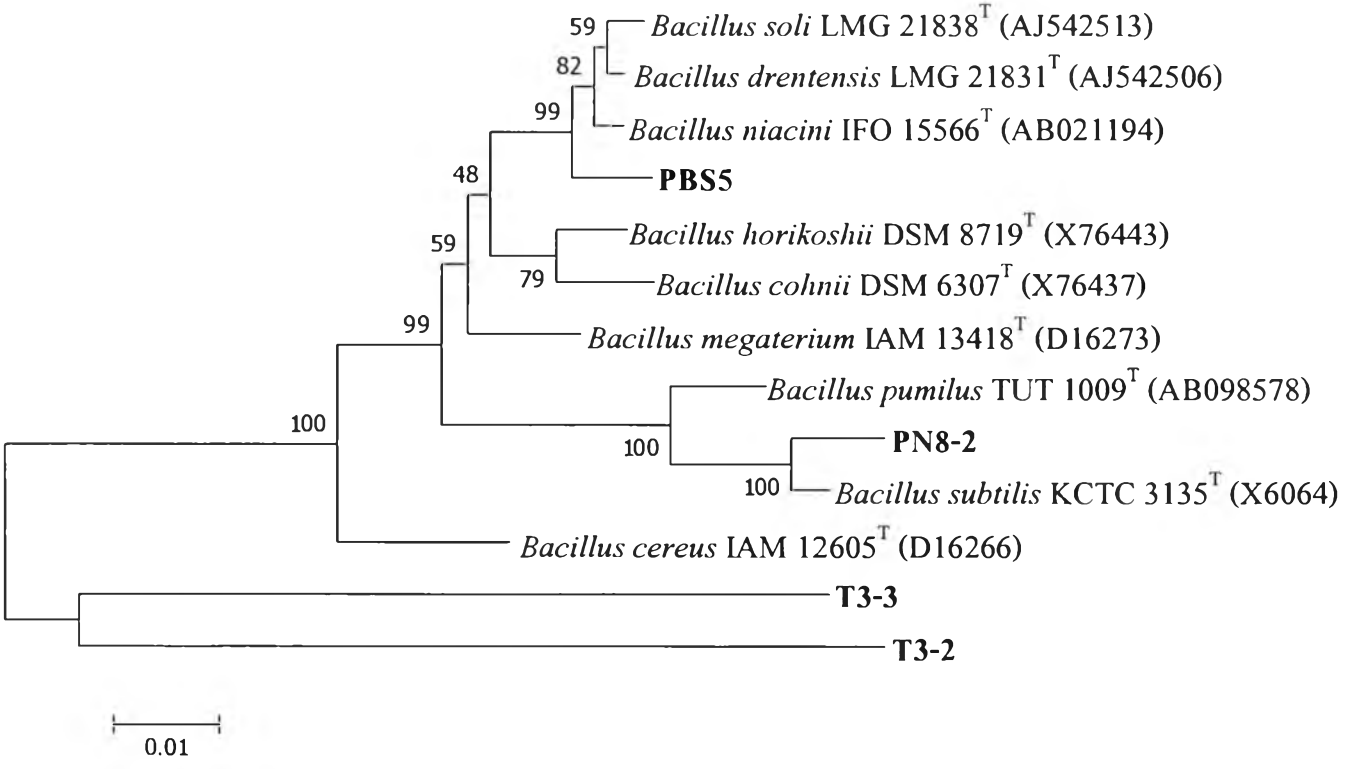

Fig. 4.7 Neighbor-joining tree showing phylogenetic position of strains PBS5, PN8-2, T3-3, T3-2 and related taxa based on 16S rRNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications.

Table 4.10 Percentage similarities of PBS5, PN8-2, T3-3, T3-2 and related *Bacillus* species

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. PBS5	100												
2. PN8-2	93.5	100											
3. T3-3	86.4	83.7	100										
4. T3-2	85.0	83.2	85.3	100									
5. *B. cereus* IAM 12605^T	95.2	93.4	85.7	88.7	100								
6. *B. soli* LMG 21838^T	98.5	94.7	86.7	85.2	95.3	100							
7. *B. drentensis* LMG 21831^T	98.7	94.3	86.8	85.6	95.3	99.5	100						
8. *B. niacini* IFO 15566^T	98.8	94.0	86.9	85.6	95.6	99.2	99.5	100					
9. *B. subtilis* KCTC 3135^T	94.0	98.8	84.0	83.3	94.3	95.1	94.8	94.4	100				
10. *B. megaterium* IAM 13418^T	97.4	94.1	88.0	85.7	95.9	97.4	97.5	97.4	94.8	100			
11. *B. pumilus* TUT 1009^T	94.3	96.9	84.3	84.3	94.7	95.0	95.1	95.0	97.6	95.8	100		
12. *B. horikoshii* DSM 8719^T	97.3	93.9	85.8	86.1	96.0	97.6	97.6	97.7	94.3	96.6	95.0	100	
13. *B. colmii* DSM 6307^T	96.8	94.1	86.0	85.9	96.0	96.8	96.8	96.9	94.6	97.5	95.7	98.3	100

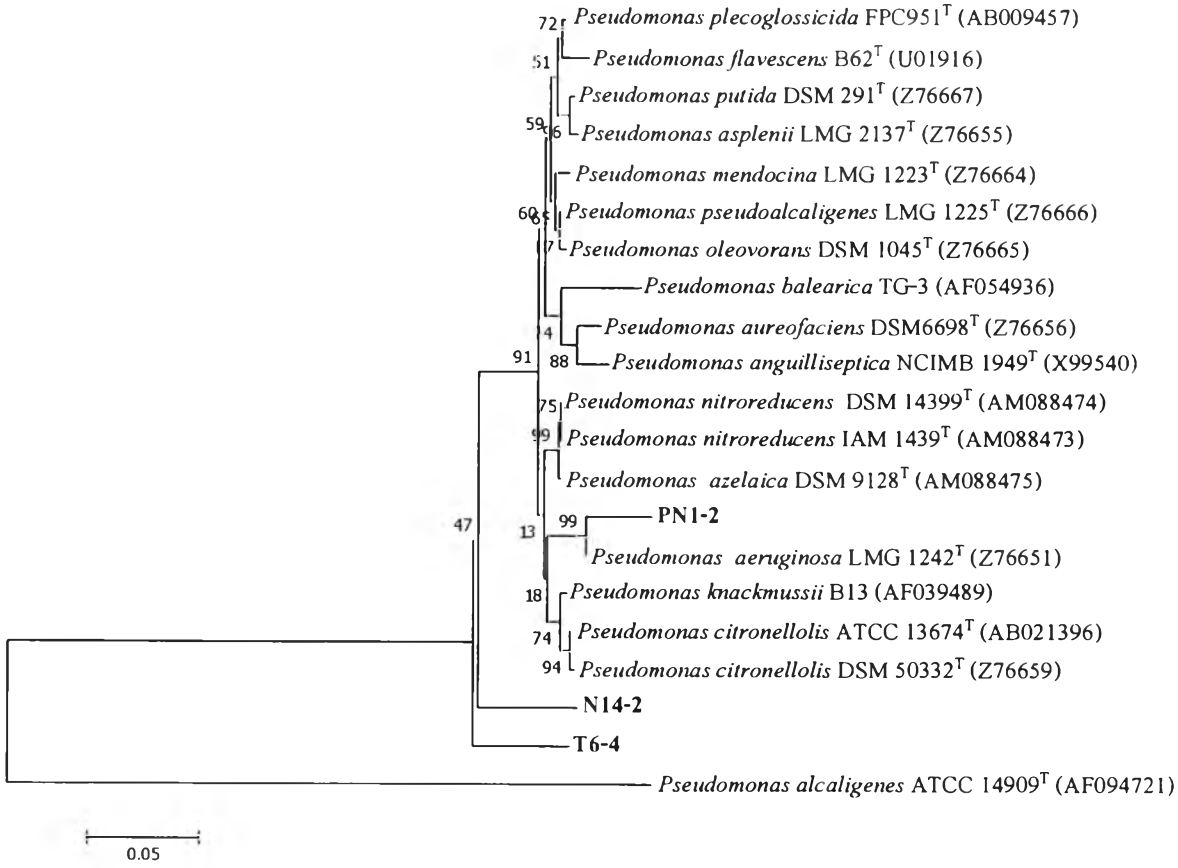

Fig. 4.8 Neighbour-joining tree showing phylogenetic position of strains PN1-2, N14-2, T6-4 and related taxa based on 16S rRNA sequences. Bar, 0.05 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications

Table 4.11 Percentage similarities of PN1-2, N14-2, T6-4, and related *Pseudomonas* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. PN1-2	100																				
2. N14-2	88.1	100																			
3. T6-4	88.3	91.0	100																		
4. *P.pseudoalcaligenes* LMG1225^T	94.0	**93.0**	91.6	100																	
5. *P.oleovorans* DSM 1045^T	93.6	92.6	91.3	99.7	100																
6. *P.mendocina* LMG1223^T	93.3	92.7	91.1	99.2	98.9	100															
7. *P.plecoglossicida* FPC951^T	93.6	91.9	91.0	99.1	99.0	98.3	100														
8. *P.putida* DSM 291^T	92.6	91.4	90.4	98.3	98.0	98.5	98.9	100													
9. *P.asplenii* LMG 2137^T	92.7	91.3	90.1	98.1	97.9	98.4	98.8	99.5	100												
10. *P.flavescens* B62^T	92.6	90.8	90.4	97.9	97.7	97.1	98.6	98.0	97.5	100											
11. *P.aureofaciens* DSM6698^T	92.0	89.8	88.4	97.1	96.8	96.7	97.4	97.6	97.8	96.3	100										
12. *P.anguilliseptica* NCIMB1949^T	93.2	89.5	89.4	96.4	96.2	96.0	95.8	96.0	96.1	94.8	97.5	100									
13. *P.aeruginosa* LMG1242^T	**97.0**	90.9	90.7	97.0	96.6	96.3	96.6	95.6	95.7	95.6	95.2	96.3	100								
14. *P.azelaica* DSM9128^T	94.3	91.2	92.5	98.0	97.8	97.4	98.0	97.4	97.2	97.2	95.7	95.5	97.3	100							
15. *P.nitroreducens* DSM14399^T	94.2	91.4	92.7	98.1	97.9	97.5	97.9	97.3	97.1	97.3	95.6	95.4	97.2	99.9	100						
16.*P.nitroreducens*IAM1439^T	94.2	91.4	**92.7**	98.1	97.9	97.5	97.9	97.3	97.1	97.3	95.6	95.4	97.2	99.9	100	100					
17. *P.knackmussii* B13^T	94.1	91.1	92.8	97.8	97.5	97.4	97.3	97.0	96.7	96.6	95.1	95.7	97.1	98.4	98.5	98.5	100				
18. *P.citronellolis* ATCC13674^T	94.7	90.6	92.5	97.5	97.2	96.8	97.2	96.5	96.3	96.3	95.2	95.9	97.6	98.5	98.4	98.4	99.3	100			
19. *P.citronellolis* DSM 50332^T	94.4	90.4	92.3	97.3	97.0	96.6	97.0	96.3	96.1	96.1	95.0	95.7	97.4	98.3	98.2	98.2	99.1	99.8	100		
20. *P.balearica* TG-3^T	91.1	88.2	88.7	94.9	94.7	94.2	94.3	94.3	94.1	93.9	94.5	94.7	94.2	94.7	94.7	94.7	95.0	94.9	94.8	100	
21. *P.alcaligenes* ATCC 14909^T	44.3	46.3	46.5	46.4	46.1	45.8	45.9	45.7	45.9	45.6	44.5	44.9	46.4	46.6	46.8	46.8	47.2	47.2	47.2	44.1	100

Characterization of the 6 groups of cellulase producing isolates

Group 1 contained 1 isolated PA4-1. It was spore forming, Gram-positive rods Colony was circular with raised margin, umbonate, translucent, off-white color. Catalase and oxidase positive. It could grow at pH 6.0-8.0 and 20-50°C but not in 5% NaCl. Negative for MR reaction, DNase, citrate utilization, TSI, hydrolysis of casein, gelation, and Tween 80 (Tables 4.7). PA4-1 (1080 bp) showed 97.2 % similarity to *Colnella thermotolerans* CCUG 47242^T. It was identified as *Colnella* (Kämpfer *et al.*, 2006) and differentiated from *Colnella thermotolerans* CCUG 47242^T by acid production from carbohydrates as shown in Table 4.12.

Table 4.12 Differential characteristics of PA4-1 in Group 1 and *C. thermotolerans* CCUG 47242^T

Characteristics	PA4-1	*C. thermotolerans* CCUG 47242^T [a]
Acid production from		
L-Arabinose	w	-
D-Cellobiose	+	-
D-Glucose	w	-
Lactose	+	-
D-Sucrose	w	-
D-Xylose	+	-

+, positive; -, negative; w, weakly positive

[a] Yoon *et al.*, 2007

Group 2 contained 3 isolates, Group 3 had 7 isolates and Group 4 had 2 isolates. They were spore forming Gram-positive bacilli. Group 2 showed circular, umbonate, entire margins, opaque, cream color. Colonies were circular, concentric, umbonate, opaque, white color in Group 3. Group 4 showed irregular and spreading, erose margins, flat, opaque, cream color colonies (Table 4.2). The representative strain, PBS5 in Group 2, T3-3 in Group 3 and T3-2 in group 4 grew in anaerobic condition and grew at 5% NaCl. No acid production from D-amygdalin, Gluconate, Glycerol, Inositol, Inulin, α–Methyl–D–glucoside, D-sorbitol, and D-sorbose. PBS5 showed positive for casein, starch hydrolysis and VP reaction but negative for citrate. T3-3 showed negative for citrate and VP reaction. T3-2 showed negative for gelatin hydrolysis, citrate and VP reaction. PBS5 (1493 bp), T3-3 (1204 BP) and T3-2 (1167 BP) showed 98.8, 88, and 88.7 % sequence similarities to *Bacillus drentensis* LMG 21831^T (Heyrman *et al.*, 2004), *Bacillus megaterium* IAM 13418^T (De Bary, 1884) and *Bacillus cereus* IAM 12605^T (Frankland and

Frankland, 1887), respectively. The character differentiated from *B. drentensis* LMG 21831^T, *B. megaterium* IAM 13418^T and *B.cereus* IAM 12605^T were shown in (Table 4.13).

Table 4.13 Differential characteristics of PBS5 in Group 2, T3-3 in Group 3, T3-2 in group 4 *B. drentensis* LMG 21831^T, *B. megaterium* IAM 13418^T and *B. cereus* IAM 12605^T

Characteristics	PBS5	T3-3	T3-2	*B. drentensis* LMG 21831$^{T\,a}$	*B. megaterium* IAM 13418$^{T\,b}$	*B. cereus* IAM 12605$^{T\,c}$
Growth at pH 6.0	+	+	+	+	v	-
Growth in 5% NaCl	+	+	+	nr	v	+
Hyhrolysis of starch	+	+	+	w	+	+
casein	+	+	+	-	+	+
Aesculin hydrolysis	-	+	+	+	nr	+
Tyrosine degradation	-	-	-	nr	v	nr
Gelatin liquefaction	-	+	-	-	nr	+
Utilization of citrate	-	-	-	-	+	+
Nitrate reduction	-	+	+	v	v	+
Voges-Proskauer reaction	+	-	-	v	-	+
Acid from D-Amygdalin	-	-	-	v	nr	-
L-Arabinose	w	+	-	-	v	-
D-Glucose	+	+	+	-	v	-
D-Mannitol	-	+	-	-	v	nr
D-Xylose	+	w	-	-	nr	-

+, positive; - , negative; w, weak positive; v, variable; nr (not report)

[a] Heyrman *et al.*, 2004; [b] De Bary, 1884; [c] Frankland and Frankland, 1887

Group 5 contained 3 isolates and Group 6 had 11 isolates. They were Gram-negative rods. Group 5 showed circular with flat and rough margins, umbonate, translucent, white cream color colonies. Colonies were circular, convex or drop-like, entire margins, translucent, no pigment in group 6 (Table 4.2). The isolates in these 2 Groups showed similar characteristics. They grew in anaerobic condition, in 5%(w/v) NaCl, pH6-9 and at 20-50° C. Positive for catalase, oxidase, nitrate reduction and Voges-Proskauer reaction (Table 4.7). No acid production from arabinose, raffinose, galactose, ribose, trehalose and xylose. The representative strain N14-2 (803

bp) in group 5 showed 93.0% sequence similarity to *Pseudomonas pseudoalcaligenes* JCM 5968^{T}. The representative strain T6-4 (800 bp) in group 6 showed 92.7% sequence similarity to *Pseudomonas nitroreducens* DSM 14399^{T}.

As mentioned above, the cellulase producing bacteria were isolates and found to be diverse species in soil samples collected, two strain of *Bacillus* and one strain of *Pseudomonas* from Tadan district, Nakhon Nayok province, one strain of *Bacillus* from Nasuan district, Suratthani province, one strain of *Pseudomonas* from Puak district, Nan province and diverse species in compost fertilizer, One strain of *Cohnella* were the novel speciesbased on phylogenetic tree analysis. Their distribution and identification were shown in Table 4.14. However, the strains that showed the 16S rRNA gene sequence similarity over 97% should be confirmed their taxonomic status by DNA-DNA hybridization experiment.

Table 4.14 Distribution and identification of the representative strains of cellulase producing isolates.

Location	Group	Isolate no.	% Similarity	Identification
Biofertilizer	1	PA4-1	97.2	*Cohnella* sp. nov
Nasuan, Suratthani province	2	PBS5	98.8	*Bacillus* sp.
Tadan, Nakhon Nayok province	3	T3-3	88	*Bacillus* sp. nov
Tadan, Nakhon Nayok province	4	T3-2	88.7	*Bacillus* sp. nov
Puak, Nan province	5	N14-2	93.0	*Pseudomonas* sp. nov
Tadan, Nakhon Nayok province	6	T6-4	92.7	*Pseudomonas* sp. nov

4.2.2 Identification of the xylanase producing bacteria

4.2.2.1 Cell morphological and cultural characteristics

On the basis of cell morphological and cultural characteristics of all 24 isolates as shown in Table 4.5, the isolates could be divided into 11 groups. One isolate in Group 1 was spore forming, Gram-positive rods. Colony was spindle with raised and entire margin, translucent, without pigmentation. One isolate in Group 2 was spore forming, Gram-positive bacilli. Colony was circular with raised and entire margin, opaque, cream color. Two isolates in Group 3 were spore forming, Gram-positive bacilli. Colonies were spindle, raised or low convex, entire margin, translucent, cream color. Two isolates in Group 4 were spore forming, Gram-positive bacilli. Colonies were circular, umbonate, entire margin, opaque, white color. Two isolates in Group 5 were Gram-positive rods. Colonies were circular, umbonate, entire margin, opaque, yellowish color. One isolate in Group 6 was Gram-positive rods. Colony was circular, umbonate, entire margins, opaque, light pink color. One isolate in Group 7 was Gram-positive rods. Colony was circular, umbonate, undulate margin, translucent, without pigment. One isolate in Group 8 was Gram-positive rods. Colony was irregular and spreading, rough, opaque, cream color. Four isolates in Group 9 were Gram-negative rods. Colonies were circular with lobate margin, translucent, green brown color. Eight isolates in Group 10 were Gram-negative rods. Colonies were circular, convex, entire margin, opaque or translucent, cream color. One isolate in Group 11 was Gram-negative rods. Colony was circular, entire margin, translucent, cream color. (Table 4.5)

4.2.2.2 Physiological and biochemical characteristics

Most of the isolates were catalase and oxidase positive. All isolates grew at pH 7-8. One strain was positive for indole production. They showed variable reaction for Methyl red, DNAase, citrate, nitrate reduction, TSI, dihydroxyacetone, gelatin hydrolysis, asculin hydrolysis, hydrolysis of L-arginine, casein, L-tyrosine, starch, and Tween 80 (Table 4.15). Acid production from carbohydrates was variable (Table 4.16 and Appendix D). All isolates grew at 20 and 45 °C, while some grew at 50 °C. The xylanase producing bacteria isolated were thermotolerant strains.

Table 4.15 Physiological and biochemical characteristics of the xylanase producing isolates.

Characteristics	Group 1 (1 isolate)	Group 2 (1 isolate)	Group 3 (2 isolates)	Group 4 (2 isolates)	Group 5 (2 isolates)	Group 6 (1 isolate)	Group 7 (1 isolate)	Group 8 (1 isolate)	Group 9 (4 isolates)	Group 10 (8 isolates)	Group 11 (1 isolate)
Growth in 5%(w/v) NaCl	-	-	-	-	-	-	-	+	+(3)	+(6)	+
Growth at pH 5.0	+	+	-	+	-	+	-	+	+	+(7)	+
pH 6.0-8.0	+	+	+	+	+(1)	+	+	+	+	+	+
pH 9.0	+	-	-	+	+(1)	-	-	+	+(2)	+(7)	+
Growth at 10 ° C	-	-	-	-	-	-	-	-	-	-	-
15 ° C	+	+	+	+	-	-	-	-	+	+	+
20 ° C	+	+	+	+	+	+	+	+	+	+	+
50 ° C	+	+	+	+	+	+	+	+	+	+(7)	+
Catalase	-	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	+	-	-	+	+	+(4)	+
Anaerobic growth	+	+	+	+	+	+	-	+	+	-(5)	+
Methyl red	-	+	-	-	-	-	-	+	-	-(7)	+
Voges-Proskauer	-	+	+	+	+	+	+	+	+	+	+

Table 4.15 (cont) Physiological and biochemical characteristics of the xylanase producing isolates.

Characteristics	Group 1 (1 isolate)	Group 2 (1 isolate)	Group 3 (2 isolates)	Group 4 (2 isolates)	Group 5 (2 isolates)	Group 6 (1 isolate)	Group 7 (1 isolate)	Group 8 (1 isolate)	Group 9 (4 isolates)	Group 10 (8 isolates)	Group 11 (1 isolate)
DNase	-	-	-	-	-	-	-	-	-	-(7)	-
Utilization of citrate	-	-	-	-	-(1)	-	-	+	+(3)	-(5)	-
TSI	N/N	N/N	N/N	N/N	N/N(1)	A/A	N/N	K/A	K/N(3)	A/A(6)	A/A
Nitrate reduction	+	+	+	+	+	+	+	+	+	-(4)	+
Aesculin	+	+	+	+	+	+	+	+	-	+(6)	-
Hydrolysis of Casein	-	-	-	-(1)	-	-	-	+	+(2)	-	-
Gelatin	+	+	-	+	+	-	+	-	+(2)	+(5)	-
Starch	+	+	+	+	+(1)	+	+	+	-	+(5)	-
Tween 80	-	-	-	-	-	-	-	-	-(2)	-(7)	-

+, positive; -, negative; N,neutral; K, alkaline; A, acid; Number in parentheses indicate the number of isolates showing the reaction.

Table 4.16 Acid from Carbohydrates of the xylanase producing isolates.

Characteristics	Group 1 (1 isolate)	Group 2 (1 isolate)	Group 3 (2 isolates)	Group 4 (2 isolates)	Group 5 (2 isolates)	Group 6 (1 isolate)	Group 7 (1 isolate)	Group 8 (1 isolate)	Group 9 (4 isolates)	Group 10 (8 isolates)	Group 11 (1 isolate)
D-Amygdalin	+	+	+	+	+(1)	+	-	-	-(3)	-(4)	-
L-Arabinose	+	+	+	+	+(1)	+	+	-	-	-(7)	+
D-Cellubiose	+	+	+	+	+(1)	-	+	-	-(3)	-(4)	-
D-Galactose	+	+	+	+	+(1)	+	+	-	-	+(6)	+
Lactose	+	+	+	+	+(1)	+	+	-	-(3)	+(4)	+
Raffinose	+	+	+	+	+(1)	+	+	-	-	-(4)	-
L-ribose	+	+	+	+	+(1)	+	+	-	-(3)	-(6)	+
Salicin	+	+	+(1)	+	+(1)	+	-	-	-	-(7)	+
D-Trehalose	+	+	+	+	+(1)	+	+	-	-(3)	-(7)	+
D-Xylose	+	+	+(1)	+	+(1)	+	-	-	-	-(6)	+

+, positive; -, negative; Number in parentheses indicate the number of isolates showing the reaction.

4.2.2.3 Chemotaxonomic characteristics

Representative strains of each 11 different groups of the xylanase producing bacteria were selected and their chemotaxonomic characteristics were determined. Six tested strains, PT4-2 in Group 1, PN12-3 in Group 3, PN13-1 in Group 5, T3-2X in Group 6, PT2-3 in Group 7, and PN8-2 in Group 8 contained *meso*-diaminopimelic acid as a diagnostic diamino in the cell wall peptidoglycan (Fig. 4.9).Five tested strains, PN8-3 in Group 2, PT6-2 in Group 4, PN1-2 in Group 9, PN9-3 in Group 10, and N9-2 in Group 11 did not contain *meso*-diaminopimelic acid as a diagnostic diamino in the cell wall peptidoglycan (Fig. 4.9). The predominant menaquinone (MK-7) was found in strains PT4-2 in Group 1, PN8-3 in Group 2, PN12-3 in Group 3, PT6-2 in Group 4, PN13-1 in Group 5, T3-2X in Group 6, PT2-3 in Group 7, and PN8-2 in Group 8. The DNA G+C contents of the tested strains ranged from 52.3-64.9 mol% as shown in Table 4.17.

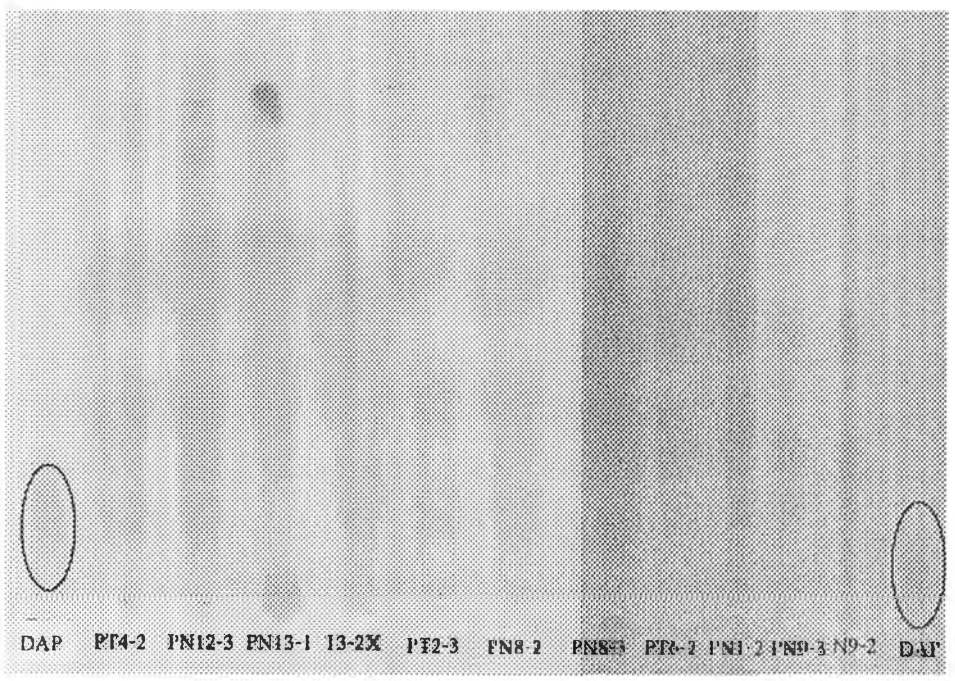

Fig. 4.9 Thin layer chromatograph showing cell wall composition of representative strains of each 11 different groups of xylanase producing isolates. (DAP: diaminopimelic acid)

Table 4.17 DNA G+C contents of the representative strains of xylanase producing isolates

Group	Representative strain	G+C content (mol%)
1	PT4-2	56.4
2	PN8-3	64.9
3	PN12-3	60.1
5	PN13-1	52.3

On the basis of their phenotypic and chemotaxonomic characteristics, Six isolates in Group 1, 2, 3, and 4 were closed to *Colnella* (Kämpfer *et al.*, 2006). Four isolates in Group 5, 6, and 7 showed characteristics that closed to *Paenibacillus* (Ash *et al.*, 1991; 1993). One isolates in Group 8 was closed to *Bacillus* (Turnbul, 1996 ; Takeuchi and Hatano, 1998 ; Venkateswaran *et al.*, 2003). Four isolates in Group 9 showed characteristics that closed to *Pseudomonas* (Anzai et al., 2000). Eight isolates in Group 10 were closed to *Acinetobacter*. And one isolate in Group 11 showed characteristics that closed to *Escherichia coli*.

4.2.2.4 16S rRNA gene sequence and phylogenetic tree analysis

The representative strain PT4-2 (1510 bp) in Group 1 and PN12-3 (1505 bp) in Group 3 showed 92.4 and 91.2 % similarity to *Colnella panacarvi* KCTC 13060^T, respectively. Strain PN8-3 (1491 bp) in Group 2 and PT6-2 (1508 bp) in Group 4 showed 91.4 and 96.4 % similarities to *Colnella thermotolerans* CCUG 47242^T, respectively (Fig. 4.6, Table 4.9). Strain PN13-1 (1468 bp) in Group 5 and T3-2X (1509 bp) in Group 6 showed 87.4 and 95.3% similarities to *Paenibacillus agarexedens* DSM 1327^T. Strain PT2-3 (1331 bp) in Group 7 showed 91.8% similarities to *Paenibacillus popilliae* ATCC 14706^T. (Fig. 4.10, Table 4.18). Strain PN8-2 (1524 bp) showed 98.8 % similarity to *Bacillus subtilis* KCTC 3135^T (Fig. 4.7, Table 4.10). Strain PN1-2 (1083 bp) in Group 9 showed 97.0 % similarity to *Pseudomonas aeruginosa* MML 2212^T (Fig. 4.8, Table 4.11). Strain PN9-3 (1563 bp) in Group 10 showed 98.4 % similarity to *Acinetobacter baumannii* ATCC 19606^T (Fig. 4.11, Table 4.19). Strain N9-2 (691 bp) in Group 11 showed 97.6 % similarity to *Escherichia coli* KCTC 2441^T (Fig. 4.12, Table 4.20).

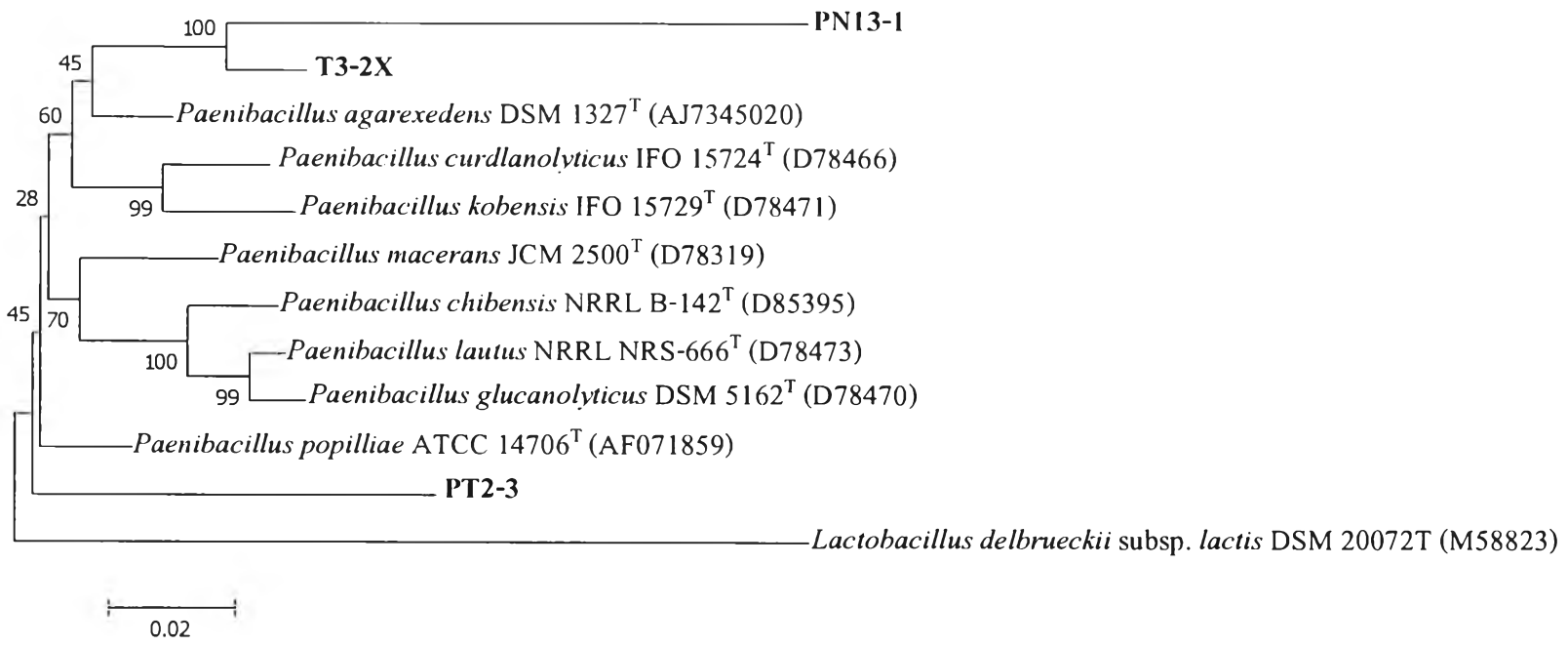

Fig. 4.10 Neighbor-joining tree showing phylogenetic position of strains PN13-1, T3-2X, PT2-3 and related taxa based on 16S rRNA gene sequences. Bar, 0.02 substitutions per nucleotide position. Bootstra p values expressed as percentages of 1000 replications.

Table 4.18 Percentage similarities of PN13-1, PT2-3, T3-2X and related *Paenibacillus* species

	1	2	3	4	5	6	7	8	9	10	11	12
1. PN13-1	100											
2. PT2-3	80.8	100										
3. T3-2X	89.6	89.8	100									
4. *P. agarexedens* DSM 1327^T	87.4	91.1	95.3	100								
5. *P. popilliae* ATCC 14706^T	86.5	91.8	94.5	96.8	100							
6. *P. curdlanolyticus* IFO 15724^T	85.6	89.9	93.4	94.6	94.3	100						
7. *P. kobensis* IFO 15729^T	85.5	89.5	93.2	94.5	94.4	96.2	100					
8. *P. macerans* JCM 2500^T	84.7	90.4	93.1	95.8	95.5	94.1	93.2	100				
9. *P. chibensis* NRRL B-142^T	84.4	90.5	92.0	94.5	94.0	92.4	92.5	94.3	100			
10. *P. lautus* NRRL NRS-666^T	84.5	89.0	92.2	94.8	94.5	92.2	91.8	94.8	97.1	100		
11. *P. glucanolyticus* DSM 5162^T	84.2	88.8	91.8	94.3	94.4	91.9	91.7	94.4	96.7	98.6	100	
12. *L.lactis* DSM 20072^T	74.8	80.7	82.0	84.2	85.4	83.9	82.6	84.2	83.8	83.4	83.2	100

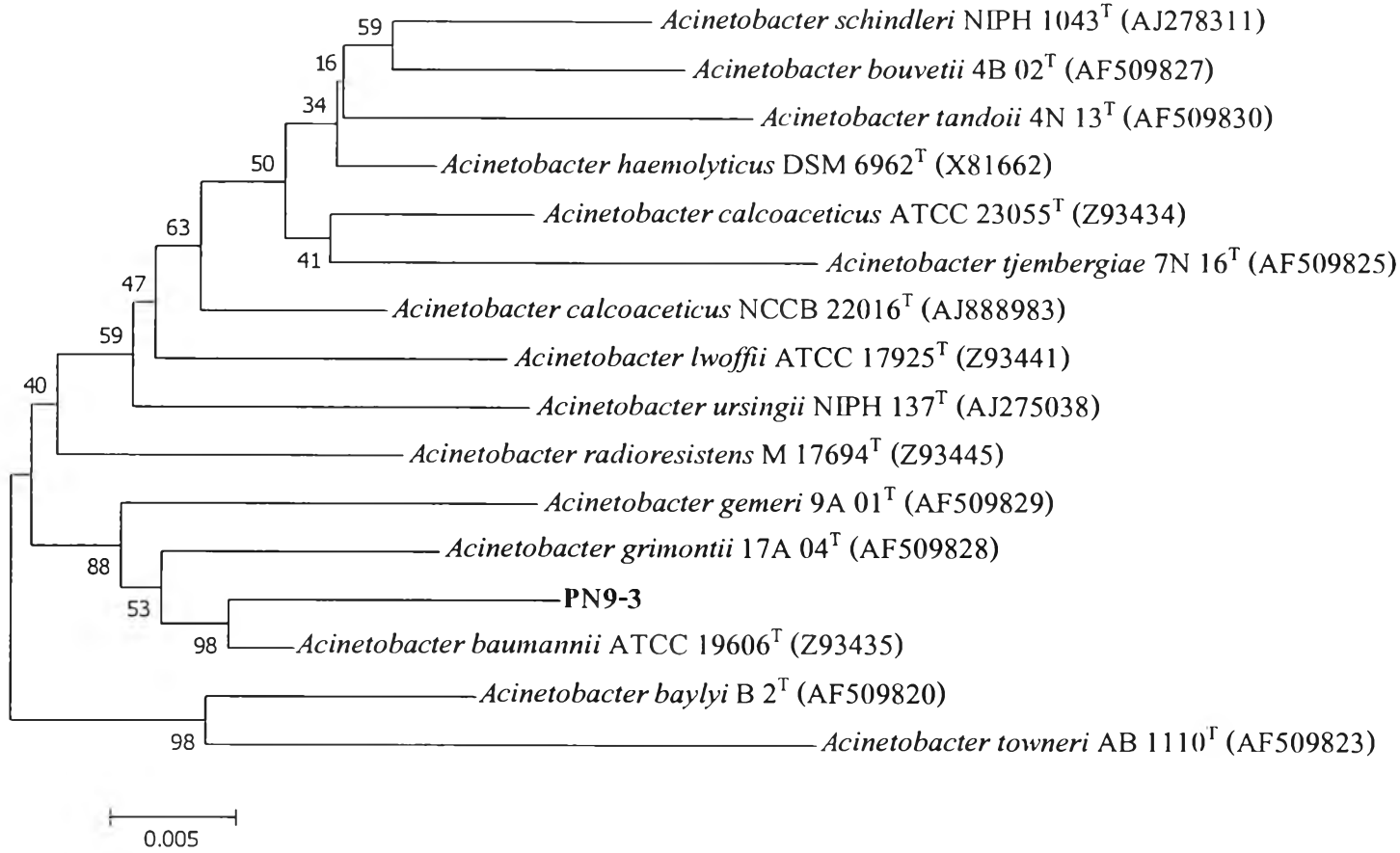

Fig. 4.11 Neighbor-joining tree showing phylogenetic position of strains PN9-3 and related taxa based on 16S rRNA gene sequences. Bar, 0.005 substitutions per nucleotide position. Bootstra p values expressed as percentages of 1000 replications.

Table 4.19 Percentage similarities of PN9-3 and *Acinetobacter* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. PN9-3	100															
2. *A. lwoffii* ATCC 17925^T	95.4	100														
3. *A.ursingii* NIPH 137^T	95.7	96.9	100													
4. *A.haemolyticus* DSM 6962^T	96.4	97.8	97.4	100												
5. *A. tandoii* 4N 13^T	95.7	96.0	96.4	98.0	100											
6. *A.schindleri* NIPH 1043^T	95.9	96.8	96.0	98.4	97.0	100										
7. *A.bouvetii* 4B 02^T	95.2	96.9	96.6	98.1	97.2	97.8	100									
8.*A.calcoaceticus*ATCC23055T	95.9	96.8	96.7	98.3	96.8	97.9	97.4	100								
9. *A.tjernbergiae* 7N 16^T	94.6	95.7	95.4	97.2	96.5	96.3	96.2	97.2	100							
10.*A.calcoaceticus* NCCB 22016^T	96.1	97.8	97.4	98.4	96.8	97.6	97.0	98.5	96.8	100						
11. *A. radioresistens* M 17694^T	96.6	97.1	96.6	97.0	96.3	96.6	96.0	96.4	95.4	97.2	100					
12. *A.baumanniii* ATCC 19606^T	98.4	96.7	96.8	97.4	96.7	96.9	97.4	96.9	95.8	97.2	97.6	100				
13. *A.grimontii* 17A 04^T	97.4	95.4	95.9	96.5	96.1	96.3	97.2	96.5	95.4	96.3	97.0	98.3	100			
14. *A.gerneri* 9A 01^T	96.4	95.2	96.0	96.4	95.4	96.2	95.9	96.2	94.4	96.1	96.6	97.3	97.3	100		
15. *A. baylyi* B 2^T	95.7	96.8	96.8	96.0	95.7	95.0	95.5	95.9	95.2	97.1	96.2	97.0	96.4	95.8	100	
16. *A.towneri* AB 1110^T	94.8	95.1	94.9	94.6	94.0	94.3	93.9	94.3	93.6	94.9	95.4	95.8	95.5	94.7	96.5	100

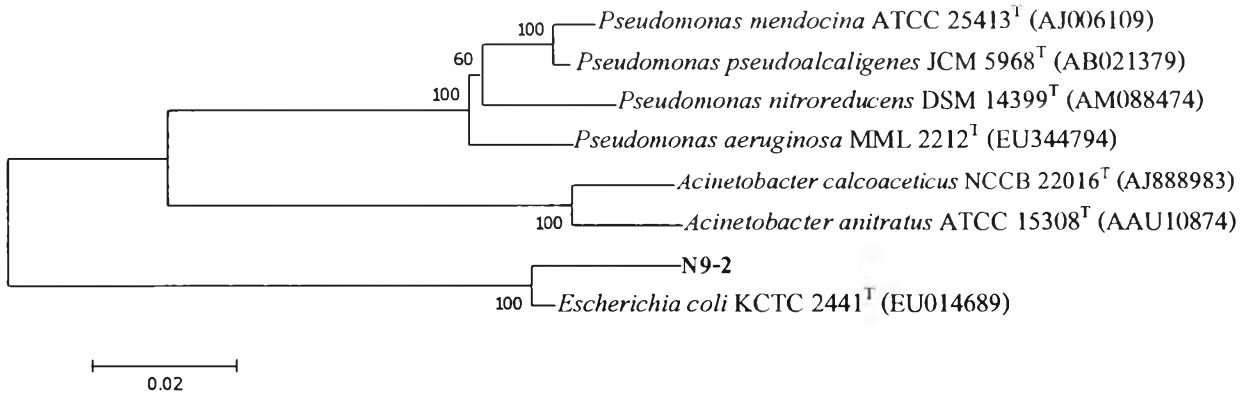

Fig. 4.12 Neighbor-joining tree showing phylogenetic position of strains N9-2 and related taxa based on 16S rRNA gene sequences. Bar, 0.02 substitutions per nucleotide position Bootstra p values expressed as percentages of 1000 replications.

Table 4.20 Percentage similarities of N9-2, *Escherichia*, *Acinetobacter* and *Pseudomonas* species.

	1	2	3	4	5	6	7	8
1. N9-2	100							
2. *E.coli* KCTC 2441	**97.6**	100						
3. *A.calcoaceticus* NCCB 22016	81.7	83.5	100					
4. *A.anitratus* ATCC 15308	81.0	82.6	97.0	100				
5. *P.mendocina* ATCC 25413	82.4	84.2	86.9	86.8	100			
6. *P.nitroreducens* DSM 14399	82.1	83.9	86.6	86.9	96.6	100		
7. *P.aeruginosa* MML 2212	82.9	84.7	86.9	87.2	96.8	96.4	100	
8. *P.pseudoalcaligenes* JCM 5968	82.7	84.5	87.4	87.2	99.2	96.8	97.3	100

Characteristics of the xylanase producing isolate

Group 1 and Group 2 each contained 1 isolate, Group 3 and Group 4 each had 2 isolates. They were spore forming Gram-positive rods. Group 1 showed spindle with raised and entire margin, translucent, without pigment. Colony was circular with raised and entire margin, opaque, cream color in Group 2. Colonies of Group 3 were spindle, raised or low convex, entire margin, translucent, cream color. Group 4 showed circular, umbonate, entire margin, opaque, white color (Table 4.5). The representative strains, PT4-2 in Group 1, PN8-3 in Group 2, PN12-3 in Group 3, and PT6-2 in Group 4 grew in anaerobic condition but not in 5%(w/v) NaCl. No acid production from gluconate, inositol, inulin, and D-sorbitol. PT4-2, PN8-3, and PN12-3 contained 56.4, 64.9 and 60.1 mol % of DNA G+C contents. PT4-2 (1510 bp) and PN12-3 (1505 bp) showed 92.4 and 91.2 % similarities to *Colnella panacarvi* KCTC 13060^T, respectively. PN8-3 (1491 bp) and PT6-2 (1508 bp) showed 91.4 and 96.4 % similarities to *Colnella thermotolerans* CCUG 47242^T, respectively. They were identified as *Colnella* (Kämpfer *et al.*, 2006) and differentiated from *Colnella panacarvi* KCTC 13060^T and *Colnella thermotolerans* CCUG 47242^T by oxidase test and acid production from carbohydrates as shown in Table 4.21.

Group 5 contained 2 isolates, Group 6 and Group 7 each had 1 isolated. They were Gram-positive rods. Group 5 showed circular, umbonate, entire margin, opaque, yellowish color. Colony was circular, umbonate, entire margin, opaque, light pink color in Group 6. Colony of Group 7 was circular, umbonate, undulate margin, translucent, without pigment (Table 4.5). The strain PN13-1 in Group 5, T3-2X in Group 6, and PT2-3 in Group 7 grew at 20-50° C but not in 5%(w/v) NaCl. Negative for citrate utilization and DNase reaction. Positive for nitrate and Voges-Proskauer reaction. No acid production from L-sorbose. PN13-1 (1468 bp) and T3-2X (1509 bp) showed 87.4 and 95.3% similarities to *Paenibacillus agarexedens* DSM 1327^T. PT2-3 (1331 bp) showed 91.8% similarities to *Paenibacillus popilliae* ATCC 14706^T. They were identified as *Paenibacillus* (Ash *et al.*, 1991; 1993). The character differentiated from *Paenibacillus agarexedens* DSM 1327^T (Uetanabaro *et al.*, 2003) and *Paenibacillus popilliae* ATCC 14706^T (Pettersson *et al.*, 1999) were shown in Table 4.22.

Table 4.21 Differential characteristics of PT4-2 (Gr.1), PN8-3 (Gr.2), PN12-3 (Gr.3), PT6-2 (Gr.4), *Colnella thermotolerans* CCUG 47242^{T} and *Colnella panacarvi* KCTC 13060^{T}

Characteristics	PT4-2	PN8-3	PN12-3	PT6-2	*C. panacarvi* KCTC 13060^{T} [a]	*C. thermotolerans* CCUG 47242^{T} [a]
Oxidase	-	-	-	-	+	+
Acid production from						
L-Arabinose	+	+	w	+	+	-
D-Cellobiose	+	+	+	+	+	-
D-Glucose	+	+	w	+	+	-
D-Maltose	+	+	+	+	+	-
D-Mannose	+	+	w	+	+	-
D-Mlibiose	+	+	+	+	+	-
Methyl α -D-glucoside	w	+	-	+	+	-
Lactose	+	+	+	+	+	-
D-Raffinose	+	+	w	+	+	-
D-Sucrose	+	+	+	+	+	-
D-Trehalose	+	+	+	+	+	-
D-Xylose	+	+	+	+	+	-
DNA G+C content (mol%)	56.4	64.9	60.1	ND	53.4	59.0

+, positive; -, negative; w, weakly positive; ND, not determined

[a] Yoon *et al.*, 2007

Table 4.22 Differential characteristics of PN13-1 (Gr.5), T3-2X (Gr.6), PT2-3 (Gr.7), *Paenibacillus agarexedens* DSM 1327^T and *Paenibacillus popilliae* ATCC 14706^T.

Characteristics	PN13-1	T3-2X	PT2-3	*P. agarexedens* DSM 1327^T [a]	*P. popilliae* ATCC 14706^T [b]
Catalase	+	+	+	+	-
Oxidase	+	-	-	+	-
Growth at 50° C	+	+	+	+	-
at pH 6.0	-	+	+	-	-
Anaerobic growth	+	+	-	-	+
Hyhrolysis of starch	+	+	+	+	-
Tween 80	-	-	-	-	nr
aesculin	+	+	+	+	nr
Gelatin liquefaction	+	-	+	-	-
Utilization of citrate	-	-	-	nr	-
Urease	-	+	-	-	nr
Nitrate reduction	+	+	+	-	-
DNase	-	-	-	-	nr
Voges-Proskauer reaction	+	+	+	-	-
Acid from Glucose	+	-	-	+	+

+, positive; - , negative; nr, not report

[a] Uetanabaro *et al.*, 2003; [b] Pettersson *et al.*, 1999

Strain PN8-2 in Group 8 was Gram-positive rods. Colony was irregular and spreading, rough, opaque, cream color. Catalase and oxidase positive. Grew in anaerobic condition, 5%(w/v) NaCl, pH6-9 and at 20-50°C. Positive for starch and casein hydrolysis, citrate utilization, nitrate reduction and Voges-Proskauer reaction. No acid production from L-arabinose, raffinose, salicin, galactose, lactose and D-xylose. PN8-2 (1524 bp) showed 98.8 % similarity to *Bacillus subtilis* KCTC 3135^T. The characters differentiated from *Bacillus subtilis* KCTC 3135^T was shown in Table 4.23.

Table 4.23 Differential characteristics of PN8-2 and *B.subtilis* KCTC 3135[T]

Characteristics	Group 8 PN8-2	*B. subtilis* KCTC 3135[T] [a]
Anaerobic growth	+	-
Gelatin liquefaction	-	+
Acid from L-Arabinose	-	+
Raffinose	-	+
Salicin	-	+
Lactose	-	+
D-Xylose	-	+

+, positive; -, negative

[a] Venkateswaran *et al.*, 2003

Group 9 contained 4 isolates. They were Gram-negative rods. Colonies were circular with lobate margin, translucent, green brown color. The representative strain, PN1-2, grew in anaerobic condition, at 20-50° C, pH6-9 and in 5%(w/v) NaCl. Positive for catalase, oxidase, gelatin and casein hydrolysis, citrate utilization, nitrate reduction and Voges-Proskauer reaction. No acid production from L-arabinose, raffinose, salicin, galactose, lactose and D-xylose. PN1-2 (1083 bp) showed 97.0 % similarity to *Pseudomonas aeruginosa* MML 2212[T].

Eight isolates in Group 10 were Gram-negative rods. Colonies were circular, convex, entire margin, opaque or translucent, cream color. The strain PN9-3 grew at 20-50° C and pH6-9. Positive for catalase, gelatin and tween80 hydrolysis, citrate utilization, and Voges-Proskauer reaction. Produced acid from D-galactose and D-mannose. PN9-3 (1563 bp) showed 98.4 % similarity to *Acinetobacter baumannii* ATCC 19606[T].

Strain N9-2 in Group 11 was Gram-negative rods. Colony was circular, entire margin, translucent, cream color (Table 4.5). Grew in anaerobic condition, at 20-50° C, pH6-9 and in 5%(w/v) NaCl. Positive for catalase, oxidase, indole production, Urease, nitrate reduction and Voges-Proskauer reaction. No acid production from D-amygdalin, D-cellobiose, inositol, inulin, D-melezitose, methyl α-D-glucoside, raffinose, L-sorbose, and sucrose. N9-2 (691 bp) showed 97.6 % similarity to *Escherichia coli* KCTC 2441[T].

As mentioned above, the xylanase producing bacteria isolate were found to be diverse species in soil samples collected, two strains of *Cohnella* and two strains of *Paenibacillus* from Tadan district, Nakhon Nayok province, two strains of *Cohnella*, one strain of *Paenibacillus*, *Bacillus*, *Pseudomonas*, and *Acinetobacter* from Nangrong district, Nakhon Nayok province, and one strain of *Escherichia* from Puak district, Nan province were the novel species based on phylogenetic tree analysis. Their distribution and identification were shown in Table 4.24. However, the strains that showed the 16S rRNA gene sequence similarity over 97% should be confirmed their taxonomic status by DNA-DNA hybridization.

Table 4.24 Distribution and identification of the representative strains of xylanase producing isolates

Location	Group	Isolate no.	% Similarity	Identification
Tadan, Nakhon Nayok province	1	PT4-2	92.4	*Cohnella* sp. nov
Nangrong, Nakhon Nayok province	2	PN8-3	91.4	*Cohnella* sp. nov
Nangrong, Nakhon Nayok province	3	PN12-3	91.2	*Cohnella* sp. nov
Tadan, Nakhon Nayok province	4	PT6-2	96.4	*Cohnella* sp. nov
Nangrong, Nakhon Nayok province	5	PN13-1	87.4	*Paenibacillus* sp. nov
Tadan, Nakhon Nayok province	6	T3-2X	95.3	*Paenibacillus* sp. nov
Tadan, Nakhon Nayok province	7	PT2-3	91.8	*Paenibacillus* sp. nov
Nangrong, Nakhon Nayok province	8	PN8-2	98.8	*Bacillus* sp.
Nangrong, Nakhon Nayok province	9	PN1-2	97.0	*Pseudomonas* sp. nov
Nangrong, Nakhon Nayok province	10	PN9-3	98.4	*Acinetobacter* sp. nov
Puka, Nan province	11	N9-2	97.6	*Escherichia*

4.3 Effect of pH and temperature on enzyme activity

4.3.1 Effect of pH and temperature on cellulase activity

Strains produced highest endoglucanase (PB1 1), highest ß-glucosidase (PA4-3), high for both endoglucanase and ß-glucosidase (PA3-3), highest HC value (PA4-1) and clear zone on cellulose powder medium (PD1-2), were chosen for this study.

The chosen strains were cultivated in CMC medium at 40° C, 200 rpm for 2 days. Supernatants obtained after centrifugation at 10,000 rpm (13,300 x g), 4° C for 15 min were used as crude enzyme. Endoglucanase and ß-glucosidase activities were analysed at various pH and temperature.

Endoglucanase :

Optimal pH for endoglucanase activity of all isolates tested was 7.5, except for strain PD1-2 which showed clear zone surrounded colony grown on cellulose powder medium. At the optimal pH 7.5, endoglucanase activity of strain PB11 was maximum at 0.056 units/ml. Optimal pH for endoglucanase activity of strain PD1-2 was 6.5. The endoglucanase activity at pH 6.5 was 0.0022 units/ml (Fig. 4.13).

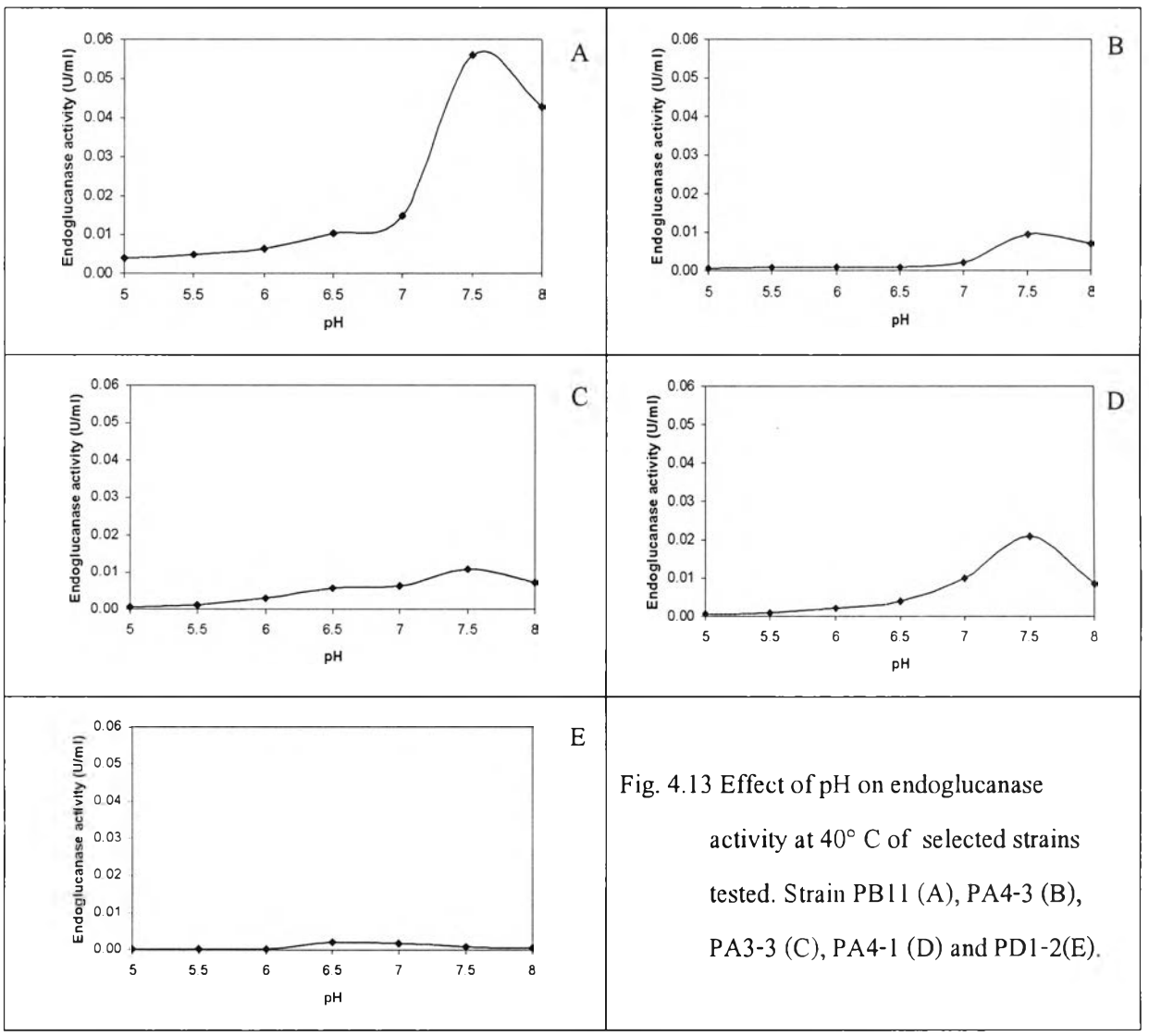

Fig. 4.13 Effect of pH on endoglucanase activity at 40° C of selected strains tested. Strain PB11 (A), PA4-3 (B), PA3-3 (C), PA4-1 (D) and PD1-2(E).

Optimum temperature for endoglucanase activity of all isolates tested was 60° C, except for strain PD1-2. At the optimal temperature (60° C), endoglucanase activity of strain PB11 was maximum at 0.11 units/ml. Optimal temperature for endoglucanase activity of strain PD1-2 was 55° C. The endoglucanase activity at 55° C was 0.016 units/ml (Fig. 4.14).

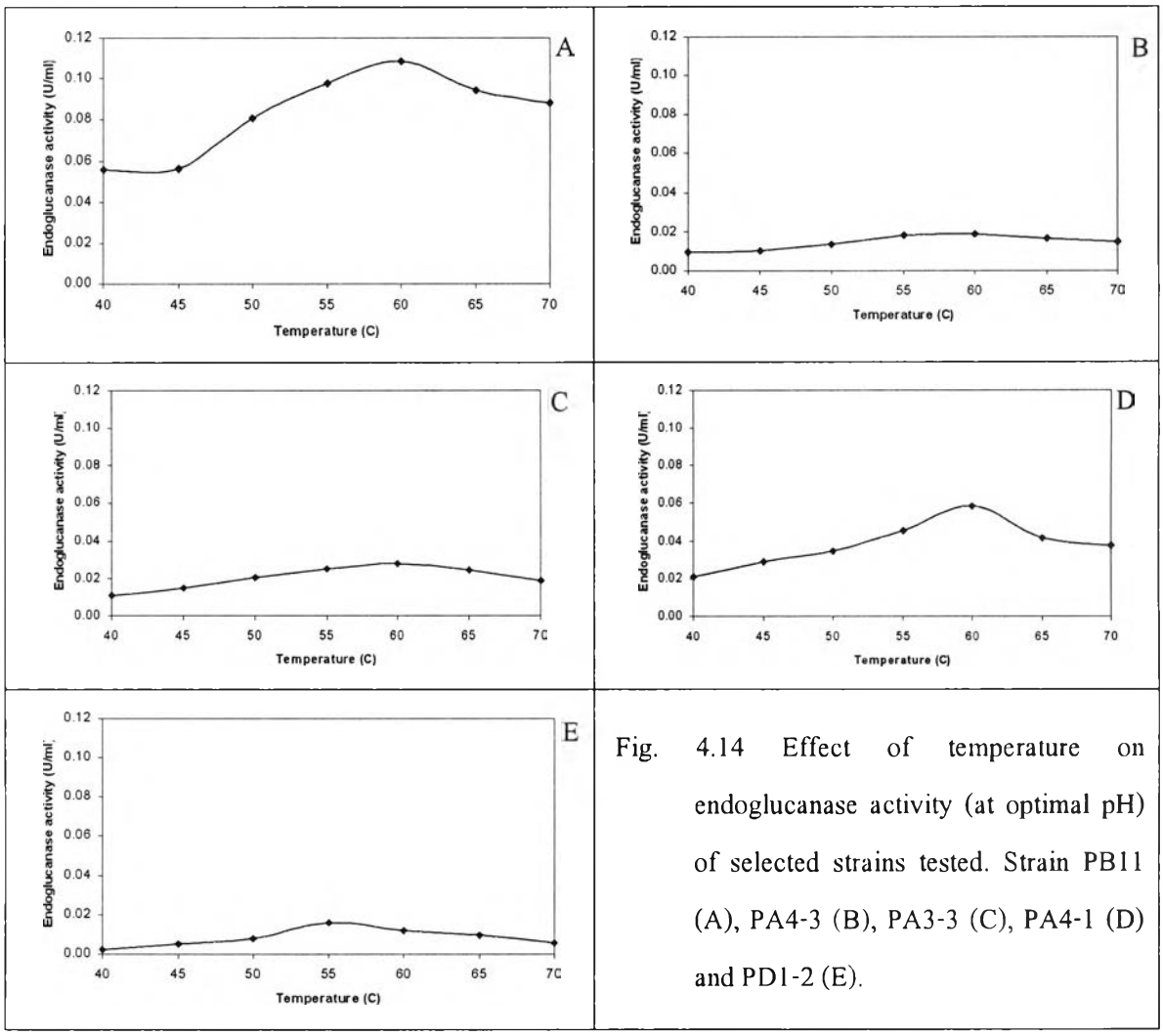

Fig. 4.14 Effect of temperature on endoglucanase activity (at optimal pH) of selected strains tested. Strain PB11 (A), PA4-3 (B), PA3-3 (C), PA4-1 (D) and PD1-2 (E).

ß-glucosidase :

Optimum pH for ß-glucosidase activity of the strains tested varied from 6-7.5. At the optimal pH, the ß-glucosidase activity ranged from 0.0014-0.0053 units/ml. Strain PA4-3 gave maximum activity at 0.0053 units/ml (Fig. 4.15).

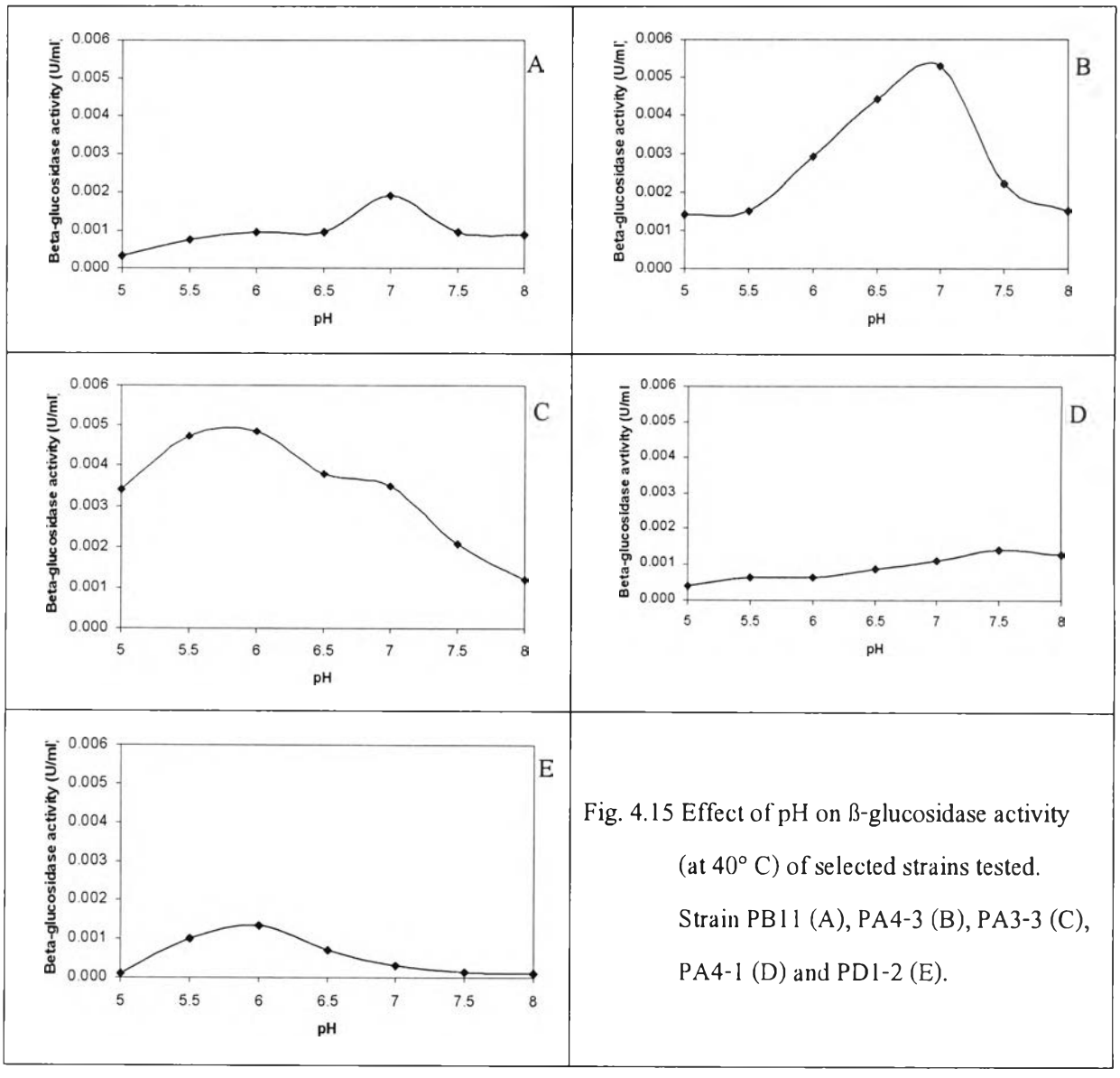

Fig. 4.15 Effect of pH on ß-glucosidase activity (at 40° C) of selected strains tested. Strain PB11 (A), PA4-3 (B), PA3-3 (C), PA4-1 (D) and PD1-2 (E).

Optimum temperature for ß-glucosidase activity of the strains tested was 60° C. At the optimal temperature, ß-glucosidase activity ranged from 0.0025-0.0091 units/ml. Strain PA4-3gave maximum activity at 0.0091 units/ml, while PD1-2 which produced clear zone on cellulose powder agar medium produced ß-glucosidase 0.0053 units/ml (Fig. 4.16).

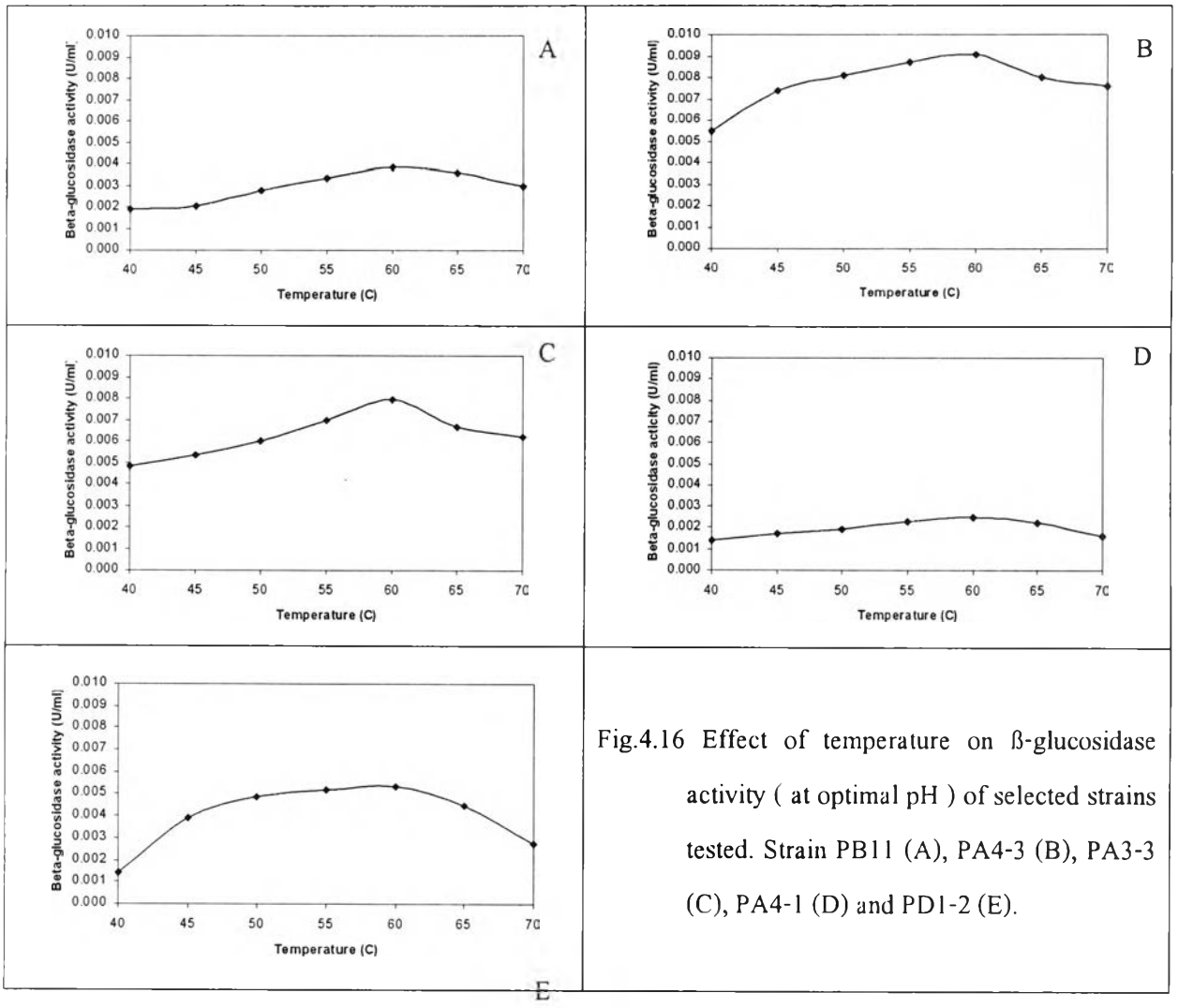

Fig.4.16 Effect of temperature on ß-glucosidase activity (at optimal pH) of selected strains tested. Strain PB11 (A), PA4-3 (B), PA3-3 (C), PA4-1 (D) and PD1-2 (E).

4.3.2 Effect of pH and temperature on xylanase activity

Top five xylanase producing strains (PN12-2, PT4-2, PT6-2, PN12-3, PN8-3) which had different phenotypic characteristics and a strain which showed highest hydrolysis capacity (HC) value, strain PN13-1, were chosen in this study.

The chosen strains were cultured in xylan medium at 40° C, 200 rpm for 2 days. Supernatants obtained after centrifugation at 10,000 rpm (13,300 x g), 4° C for 15 min were used as crude enzyme. Xylanase activity was analysed at various pH and temperature.

Optimal pH for xylanase activity of the strains tested varied from 6.5-8.0. Xylanase activity at the optimal pH ranged from 0.06-0.48 units/ml. Strain PN12-2 showed maximum xylanase activity (0.48units/ml) at pH 8.0. Strain PN13-1 which produced highest HC value showed maximum xylanase activity (0.06 units/ml) at pH 7 (Fig. 4.17).

Optimal temperature for xylanase activity of the strains tested varied from 50-65°C. Xylanase activity at the optimal temperature ranged from 0.08-0.51 units/ml. Strain PN12-2 showed maximum xylanase activity (0.51 units/ml) at 65° C. At this temperature, the xylanase activity of strain PT4-2 and PN12-3 significantly increased to 0.4 and 0.35 units/ml. The same as strain PN13-1, the xylnase activity at optimal temperature (60° C) increased to 0.17 units/ml (Fig. 4.18).

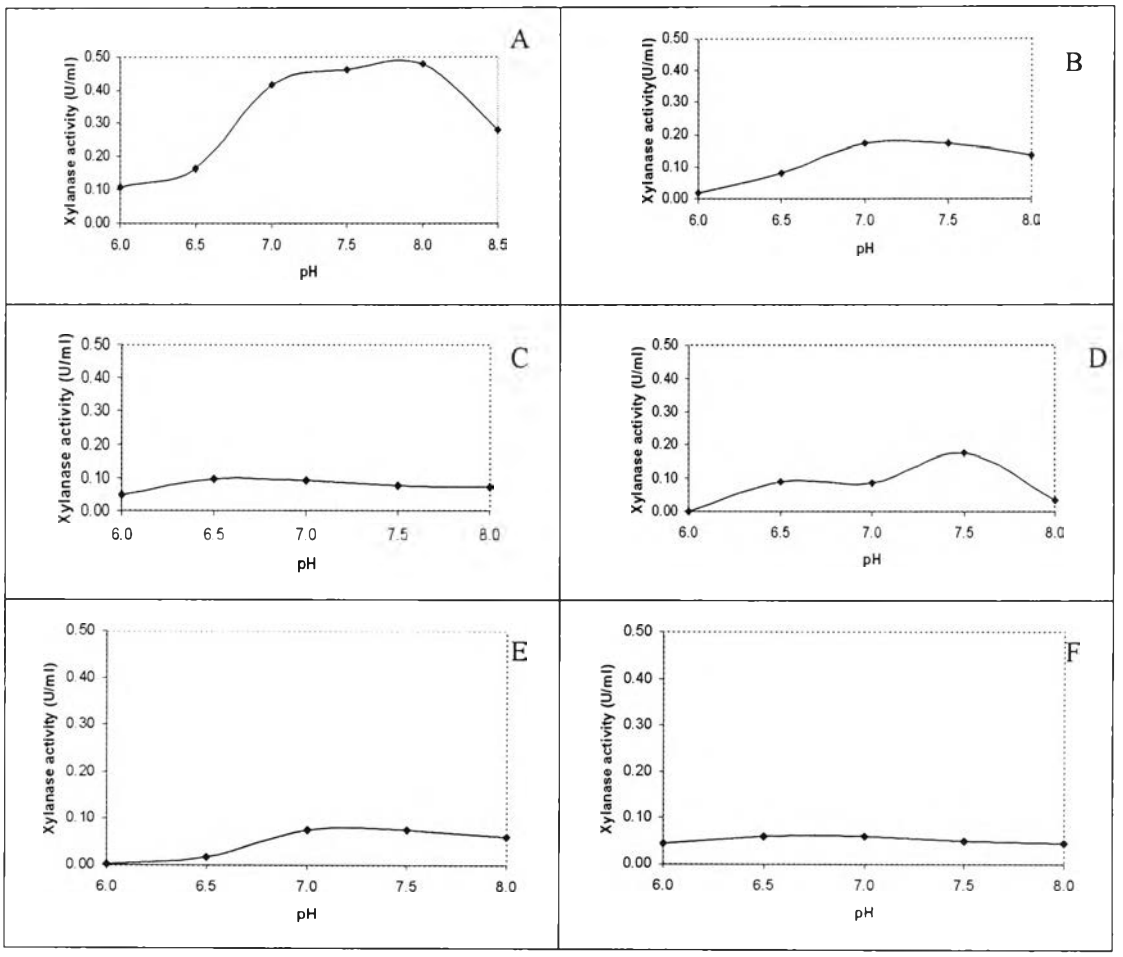

Fig. 4.17 Effect of pH on xylanase activity (at 40° C) of selected strains tested. Strain PN12-2 (A), PT4-2 (B), PT6-2 (C), PN12-3 (D) and PN8-3 (E) and PN13-1 (F).

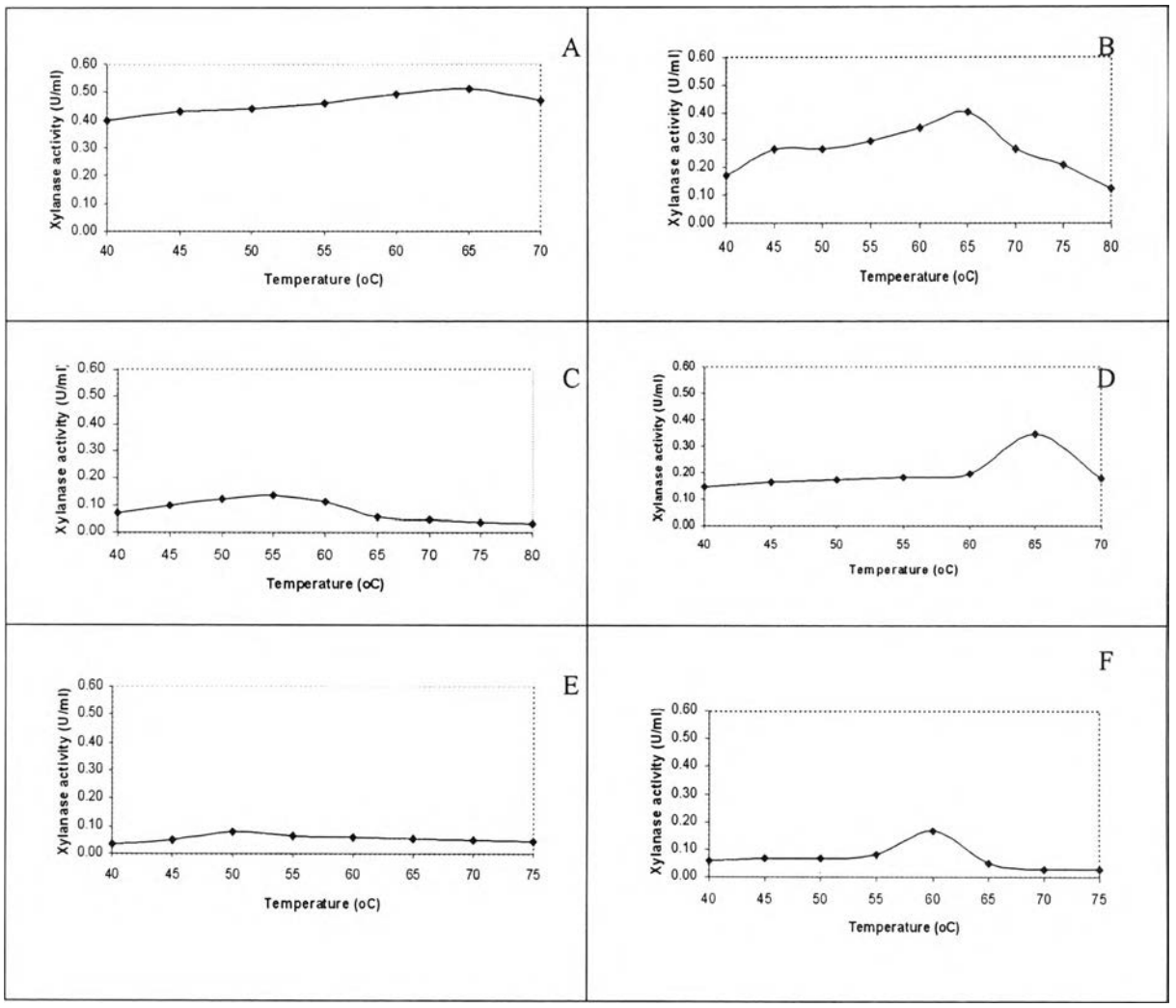

Fig. 4.18 Effect of temperature on xylanase activity (at optiaml pH) of selected strains tested. Strain PN12-2 (A), PT4-2 (B), PT6-2 (C), PN12-3 (D) and PN8-3 (E) and PN13-1 (F).

4.4 Synergistic filter paper degradation by mixed-culture of selected strains

The highest endoglucanase producing (PB11), highest ß-glucosidase producing (PA4-3), highest HC value producing (PA4-1), high for both endoglucanase and ß-glucosidase producing (PA3-3) strains and the strain produced clear zone when grown on cellulose powder medium (PD1-2) were co-cultivated as shown in Table 4.25. In PY medium (30 ml) containing 1 x 6 cm filter paper strip as single carbon source at 40° C (200 rpm) for 1 mount.

Cocultivation of the above five strains resulted in highest filter paper degradation. Dry weight lost of the filter paper was 0.0027 mg (Table 4.25, Fig. 4.19). This indicated an important of product inhibition and synergistic action of cellulase system.

Table 4.25 Synergistic filter paper degradation by mixed-culture of selected strains

Strain					Dry wt. loss
PB11	PA4-3	PA4-1	PA3-3	PD1-2	(mg)
0	0	0	0	0	0
1	0	0	0	0	0.0010
0	1	0	0	0	0
0	0	1	0	0	0.0017
0	0	0	1	0	0.0006
0	0	0	0	1	0.0008
1	1	1	1	1	**0.0027**

0, absence; 1, presence

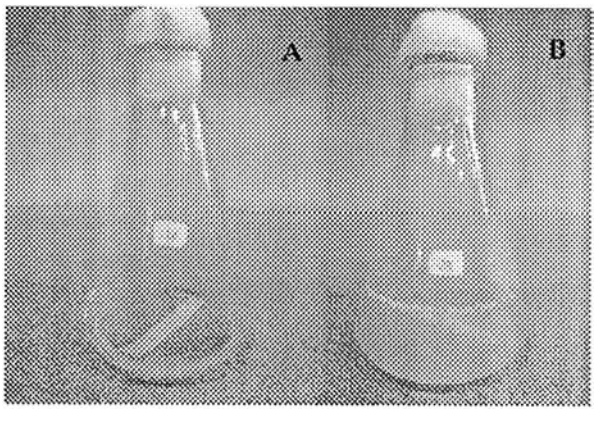

Fig. 4.19 Filter paper degradation after for 1 month. Control (A), mixed-culture of PB11, PA4-3, PA4-1, PA3-3 and PD1-2 (B).