CHAPTER IV

RESULTS AND DISCUSSION

1. Screening of cellulase producing bacteria

1.1 Screening of cellulase producing bacteria on agar plate

The cellulase-producing bacteria were isolated from 120 soil samples collected in Pua, and Santisuk districts, Nan province, Thailand, by using enrichment culture method and incubating at 40 ^{o} C. Seventy-five cellulase producers produced clearance zone surround their colonies grown on carboxymethyl cellulose basal agar medium were isolated (Table 4.1).

Table 4.1 Sample location , isolate number, and number of isolates obtained

Location	Isolate no.	No. of isolate
Pua district :	P1-1, P1-2, P1-3, P1-4, P1-5, P1-6, P1-7, P1-8, P1-9, P1-10, P1-11, P1-12, P2-1, P2-2, P2-3, P2-4, P2-5, P3-1, P3-2, P3-3, P3-4, P3-5, P4-1, P4-2, P4-3, P4-4, P4-5, P4-6, P4-7, P4-8, P4-9, P4-10, P4-11, P4-12, P4-13, P5-1, P5-2, P5-3, P5-4, P5-5, P5-6, P5-7, P5-8, P5-9, P5-10, P5-11, P5-12, P5-13, P5-14, P6-1, P6-2, P6-3, P6-4, P6-5, P6-6, P6-7, P6-8, P6-9, P6-10, P7-1, P7-2, P7-3, P7-4, P7-5, P7-6, P7-7	66
Santisuk district :	S8-1, S8-2, S8-3, S9-2, S10-1, S10-2, S10-3, S10-4, S11-1	9
Total		**75**

Based on their cell morphology and cultural characteristics, the 75 isolates were into 9 groups as shown in Table 4.2. Cellulolytic activity on agar medium (clearance zone diameter) of each isolates grown on CMC-basal agar at 40 ^{0} C for 2 days and hydrolysis capacity (HC) value calculated from the clearance zone diameter (cm) divided by colony diameter (cm) were shown in Table 4.3.

1.2 Cellulase production

Cellulase production of 52 isolates ranged from 0-0.005 units/ml; 20 isolates was 0.005-0.1 units/ml, and 3 isolates was more than 0.01 units/ml. Strain P3-1 in Group 5 produced a maximum cellulase at 0.0153 units/ml. Cellulase production of all isolates grown in CMC medium was shown in Fig 4.2- 4.5.

2. Identification

2.1 Cell morphology and cultural characteristics

On the basis of cell morphology and cultural characteristics of all 75 isolates as shown in Table 4.2, the isolates could be divided into 9 groups. Two isolates in Group 1 were spore forming, Gram-positive rods. Colonies were circular, raised, entire margins, smooth, translucent, white colour. Nine isolates in Group 2 were spore forming, Gram-positive rods. Colonies were circular, raised, entire margins, smooth, dull, white cream colour. Twenty-four isolates in Group 3 and 4 were Gram–positive bacilli. Colonies were circular/irregular, raised, entire /undulate margins, smooth, dull, white colour. Twelve isolates in Group 5 were Gram-positive bacilli. Colonies were circular, flat, undulate margins, smooth, dull, white colour. Twenty-one isolates in Group 6 were Gram-positive bacilli. Colonies were circular, raised, entire margins, smooth, dull, viscid, white colour. Six isolates in Group 7 and 8 were Gram-positive bacilli. Colonies were irregular, raised, lobate margins, rough, dull, yellow and yellowish white colour. An isolate in Group 9 was Gram -positive bacilli. Colonies were circular, raised, undulate margins , wrinkled, dull, white colour (Table 4.2).

Cells of a high cellulase production strain P4-6 grown on carboxymethyl cellulose medium at 37 $^{\circ}$ C for 2 day were shown in Fig 4.1.

Table 4.2 Cell morphology and cultural characteristics of the isolates.

Isolate no.	Colony morphology	Cell shape	Gram	Endospore
Group1: P5-5	Circular, raised, entire margins,	Rods	+	+
P6-5	smooth, translucent, white colour	Rods	+	+
	colonies			
Group2: P1-4	Circular, raised, entire margins,	Rods	+	+
P1-9	smooth, dull, white cream colour	Rods	+	+
P2-1	colonies	Rods	+	+
P3-2		Rods	+	+
P5-7		Rods	+	+
P5-8		Rods	+	+
P6-6		Rods	+	+
S8-1		Rods	+	+
S10-2		Rods	+	+
Group3: P1-2	Circular, raised, entire margins,	Rods	+	+
P1-3	smooth, dull, white colour	Rods	+	+
P2-2	colonies	Rods	+	+
P6-2		Rods	+	+
P6-3		Rods	+	+
P7-4		Rods	+	+
P7-5		Rods	+	+
P7-6		Rods	+	+
P7-7		Rods	+	+
S8-4		Rods	+	+
S10-4		Rods	+	+

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

Isolate no.	Colony morphology	Cell shape	Gram	Endospore
Group4: P1-7	Irregular, raised,	Rods	+	+
P1-11	undulate margins , smooth, dull,	Rods	+	+
P2-3	white colour colonies	Rods	+	+
P4-6		Rods	+	+
P4-7		Rods	+	+
P4-11		Rods	+	+
P5-3		Rods	+	+
P5-6		Rods	+	+
P6-4		Rods	+	+
P6-9		Rods	+	+
P6-10		Rods	+	+
P7-2		Rods	+	+
S10-3		Rods	+	+
Group5: P1-1	Circular, flat, undulate margins,	Rods	+	+
P4-1	smooth, dull, white colour	Rods	+	+
P4-3	colonies	Rods	+	+
P4-4		Rods	+	+
P4-5		Rods	+	+
P4-9		Rods	+	+
P4-10		Rods	+	+
P5-1		Rods	+	+
S8-3		Rods	+	+
S9-2		Rods	+	+
S10-1		Rods	+	+
S11-1		Rods	+	+

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

Isolate no.	Colony morphology	Cell shape	Gram	Endospore
Group6: P1-6	Circular, raised, entire margins,	Rods	+	+
P1-8	smooth, dull, viscid, white colour	Rods	+	+
P1-10	colonies	Rods	+	+
P2-4		Rods	+	+
P2-5		Rods	+	+
P3-1		Rods	+	+
P3-4		Rods	+	+
P3-5		Rods	+	+
P4-2		Rods	+	+
P4-12		Rods	+	+
P4-13		Rods	+	+
P5-4		Rods	+	+
P5-9		Rods	+	+
P5-10		Rods	+	+
P5-11		Rods	+	+
P5-12		Rods	+	+
P5-13		Rods	+	+
P5-14		Rods	+	+
P6-1		Rods	+	+
P6-8		Rods	+	+
S8-2		Rods	+	+

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

Isolate no.	Colony morphology	Cell shape	Gram	Endospore
Group7: P3-3	Irregular, yellowish, rough, dull,	Rod	+	+
P4-8	no pigmentation,	Rod	+	+
P5-2	raised, lobate margins	Rod	+	+
P7-1		Rod	+	+
P7-3		Rod	+	+
Group8: P1-5	Circular, yellow, smooth, dull, pigmentation, raised, entire margins	Rod	+	+
Group9: P6-7	Circular, dull white, wrinkled, dull, no pigmentation, raised, undulate margins	Rod	+	+

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: P5-5	0.24	0.63	2.63
P6-5	0.31	0.57	1.84
Group2: P1-4	0.32	0.55	1.72
P1-9	0.32	1.65	5.16
P2-1	0.25	1.70	**6.8**
P3-2	0.70	2.73	3.9
P5-7	0.38	1.63	4.29
P5-8	0.22	1.18	5.36
P6-6	0.72	2.82	3.92
S8-1	0.43	0.75	1.74
S10-2	0.31	0.51	1.65
Group3: P1-2	1.12	2.60	2.32
P1-3	0.64	1.85	2.89
P2-2	1.30	2.98	2.29
P6-2	1.50	2.75	1.83
P6-3	0.35	1.90	5.43
P7-4	2.18	3.39	1.56
P7-5	1.12	2.70	2.41
P7-6	1.35	2.40	1.78
P7-7	0.28	1.97	**7.04**
S8-4	0.51	2.11	4.14
S10-4	0.31	2.34	7.55

Table 4.3 (Cont) Cellulolytic activity of the isolates on agar medium.

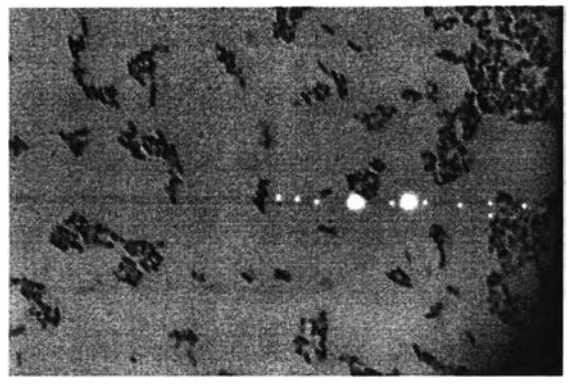
Isolate no.	CMC-basal agar medium		
	colony diameter (cm)	Clear zone diameter (cm)	HC value
Group4: P1-7	1.90	3.50	1.84
P1-11	0.70	2.60	3.71
P2-3	0.28	2.38	**8.5**
P4-6	0.35	2.00	5.71
P4-7	0.60	2.65	4.42
P4-11	0.84	2.06	2.45
P5-3	0.55	1.88	3.42
P5-6	0.30	1.52	5.07
P6-4	0.6	2.30	3.83
P6-9	1.31	2.10	1.60
P6-10	0.43	1.63	3.79
P7-2	0.22	1.43	6.5
S10-3	0.78	2.48	3.18
Group5: P1-1	1.09	1.70	1.26
P4-1	0.86	1.77	2.06
P4-3	1.32	1.74	1.32
P4-4	1.17	1.59	1.36
P4-5	1.33	1.78	1.34
P4-9	1.31	1.60	1.22
P4-10	1.43	1.79	1.25
P5-1	1.57	2.00	1.27
S8-3	1.49	1.98	1.33
S9-2	0.30	0.70	2.33
S10-1	1.35	1.55	1.15
S11-1	1.06	1.73	1.63

Table 4.3 (Cont) Cellulolytic activity of the isolates on agar medium.

Isolate no.	CMC-basal agar medium		
	colony diameter (cm)	Clear zone diameter (cm)	HC value
Group6: P1-6	1.90	3.80	2.0
P1-8	1.80	3.60	2.0
P1-10	1.49	3.03	2.03
P2-4	2.45	3.42	1.40
P2-5	1.90	3.42	1.80
P3-1	1.05	2.95	2.81
P3-4	1.90	3.05	1.61
P3-5	1.88	3.18	1.69
P4-2	1.74	3.27	1.88
P4-12	2.00	3.15	1.58
P4-13	1.76	3.06	1.74
P5-4	0.63	2.72	4.32
P5-9	0.72	2.75	3.82
P5-10	1.95	3.16	1.62
P5-11	2.10	3.15	1.50
P5-12	2.13	3.20	1.50
P5-13	2.07	3.25	1.57
P5-14	1.90	3.70	1.95
P6-1	0.60	2.70	4.5
P6-8	0.90	4.10	4.56
S8-2	1.15	1.60	1.39

Table 4.3 (Cont) Cellulolytic activity of the isolates on agar medium.

Isolate no.	CMC-basal agar medium		
	colony diameter (cm)	Clear zone diameter (cm)	HC value
Group7: P3-3	0.25	1.48	5.92
P4-8	0.24	1.45	6.04
P5-2	0.32	1.45	4.53
P7-1	0.25	1.84	7.36
P7-3	0.51	1.58	3.10
Group8: P1-5	0.49	1.44	2.94
Group9: P6-7	0.35	2.18	6.23



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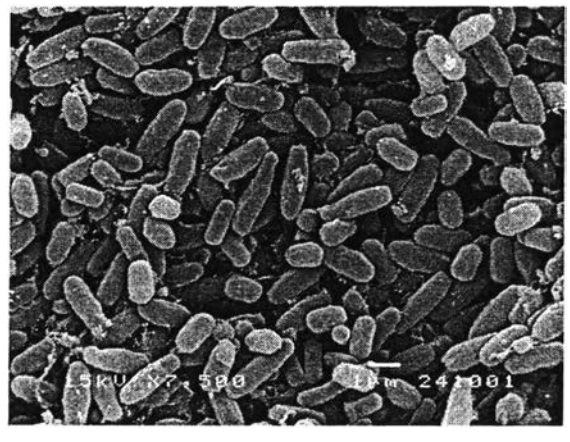

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Fig. 4.1 Photomicrograph (a) and scanning electron micrograph (b) of P4-6 grown on carboxymethyl cellulose medium at 37 $^{\circ}$ C for 2 day.

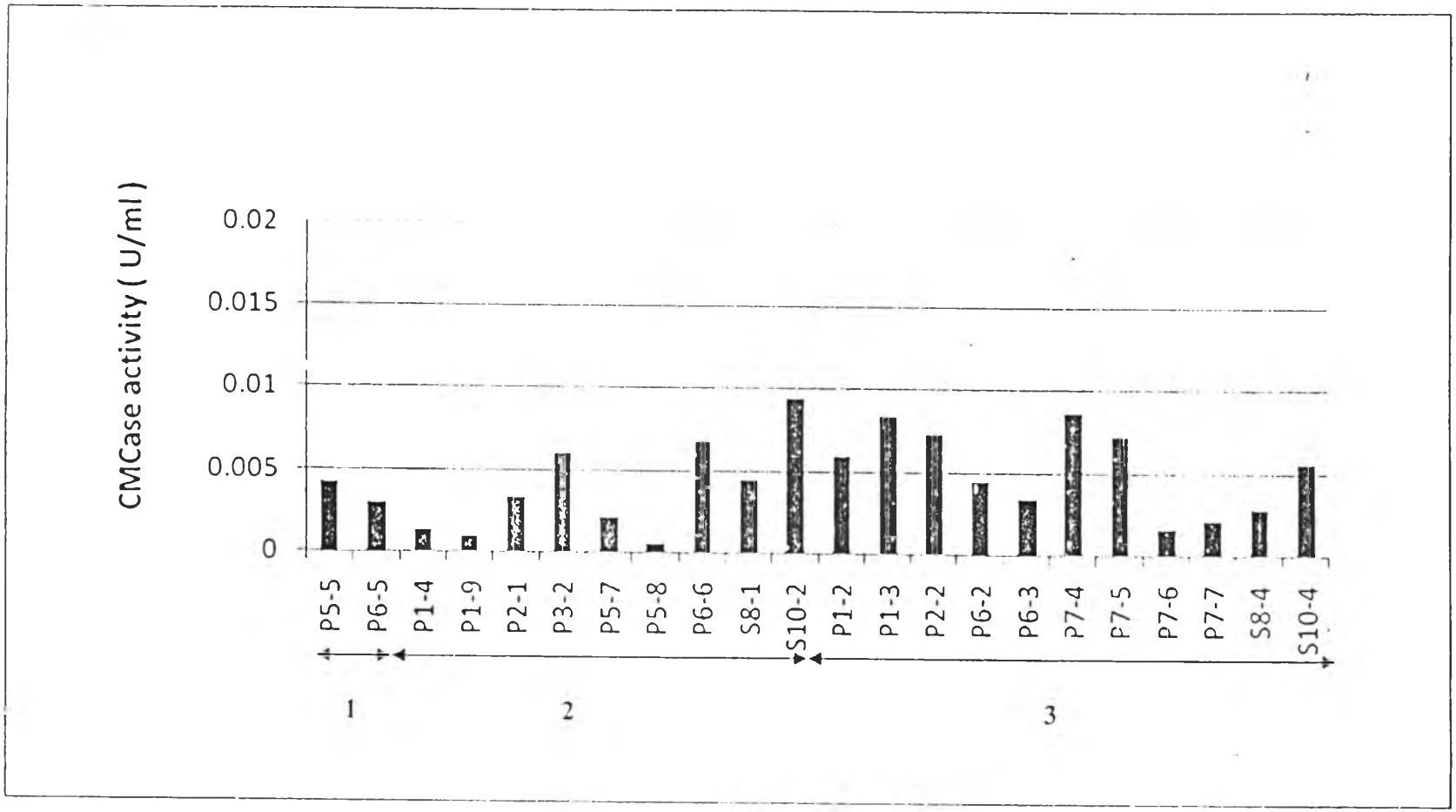


Fig 4.2 Cellulase production of the isolates in Group1, 2, and 3

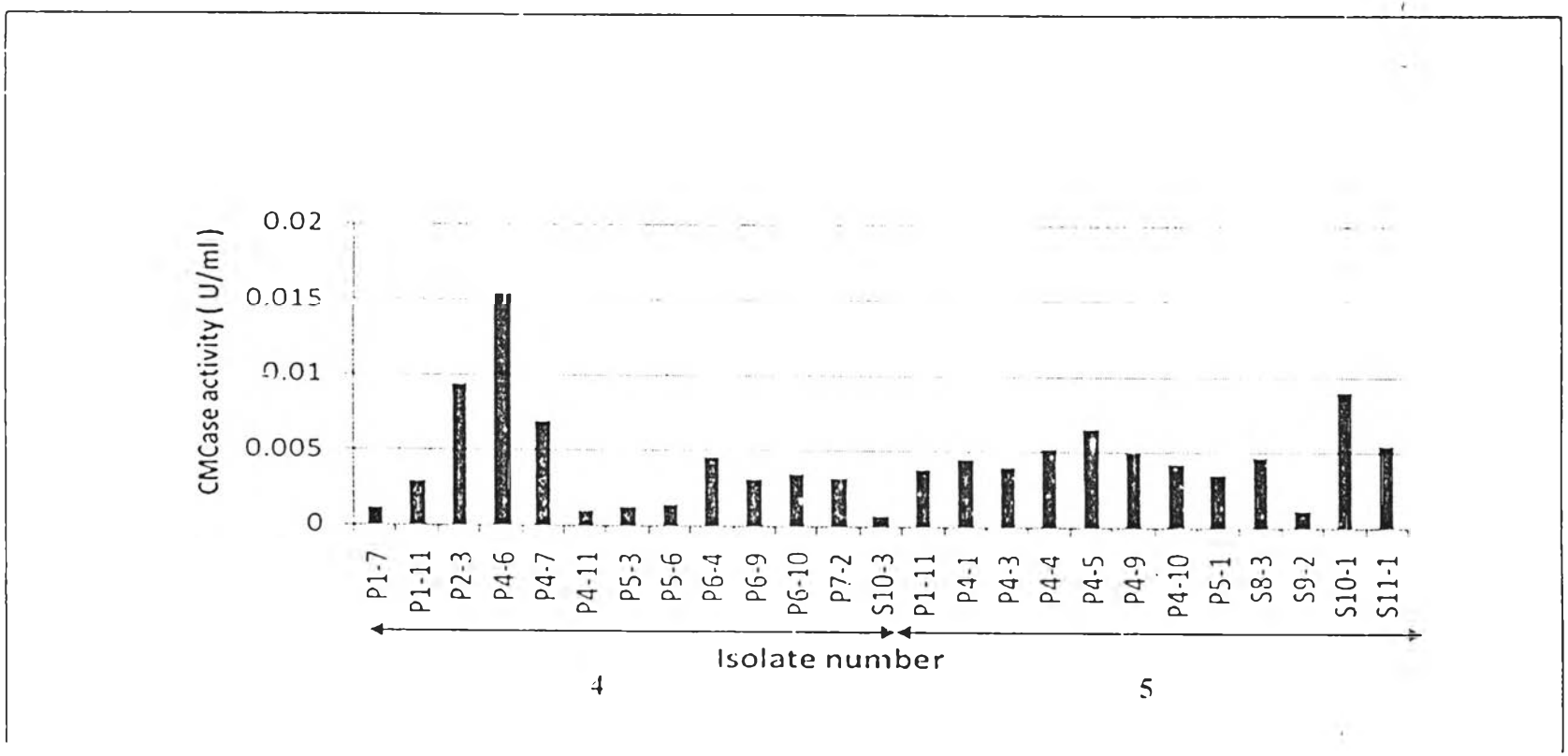


Fig 4.3 Cellulase production of the isolates in Group 4 and 5

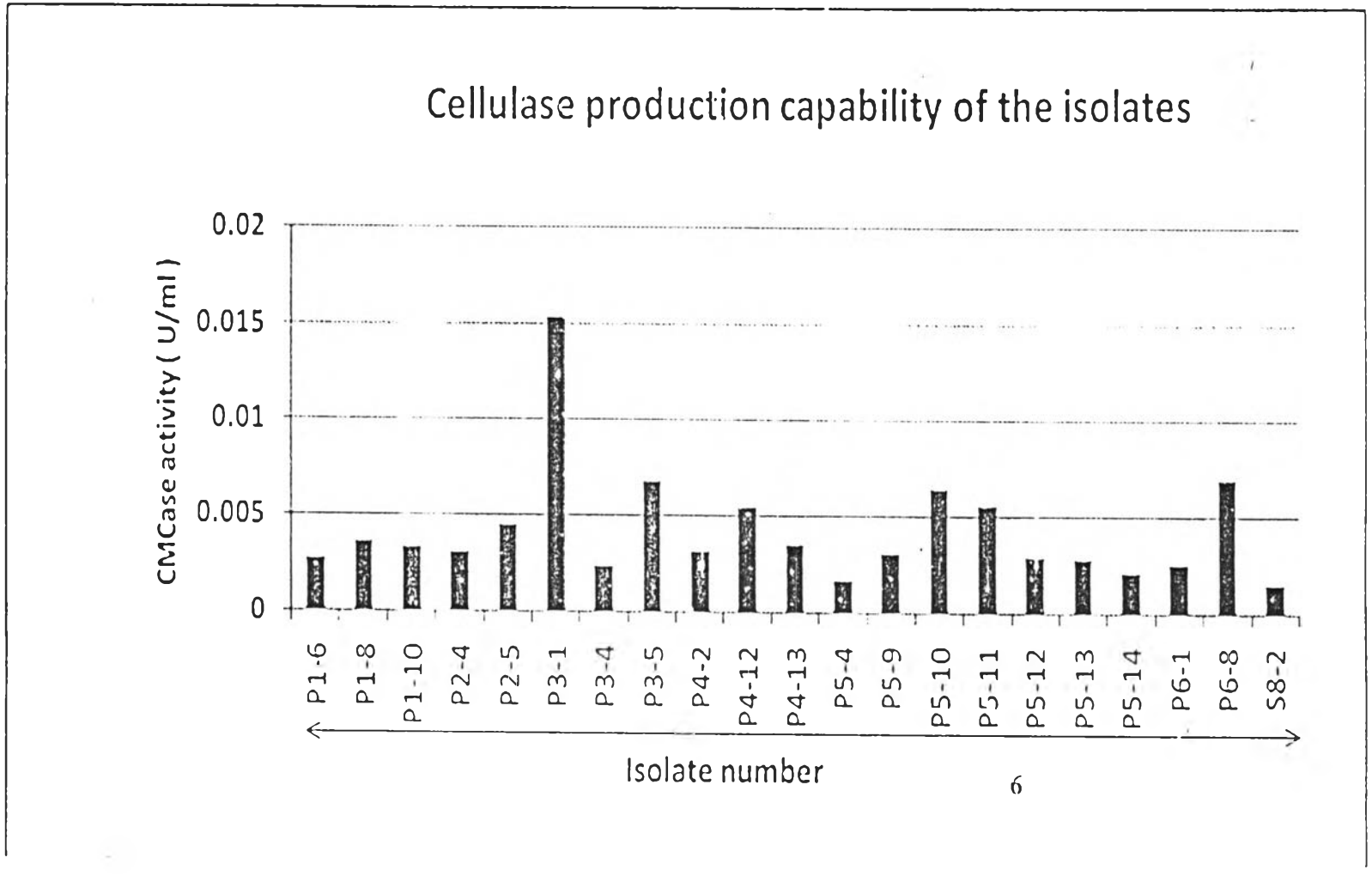


Fig 4.4 Cellulase production of the isolates in Group 6

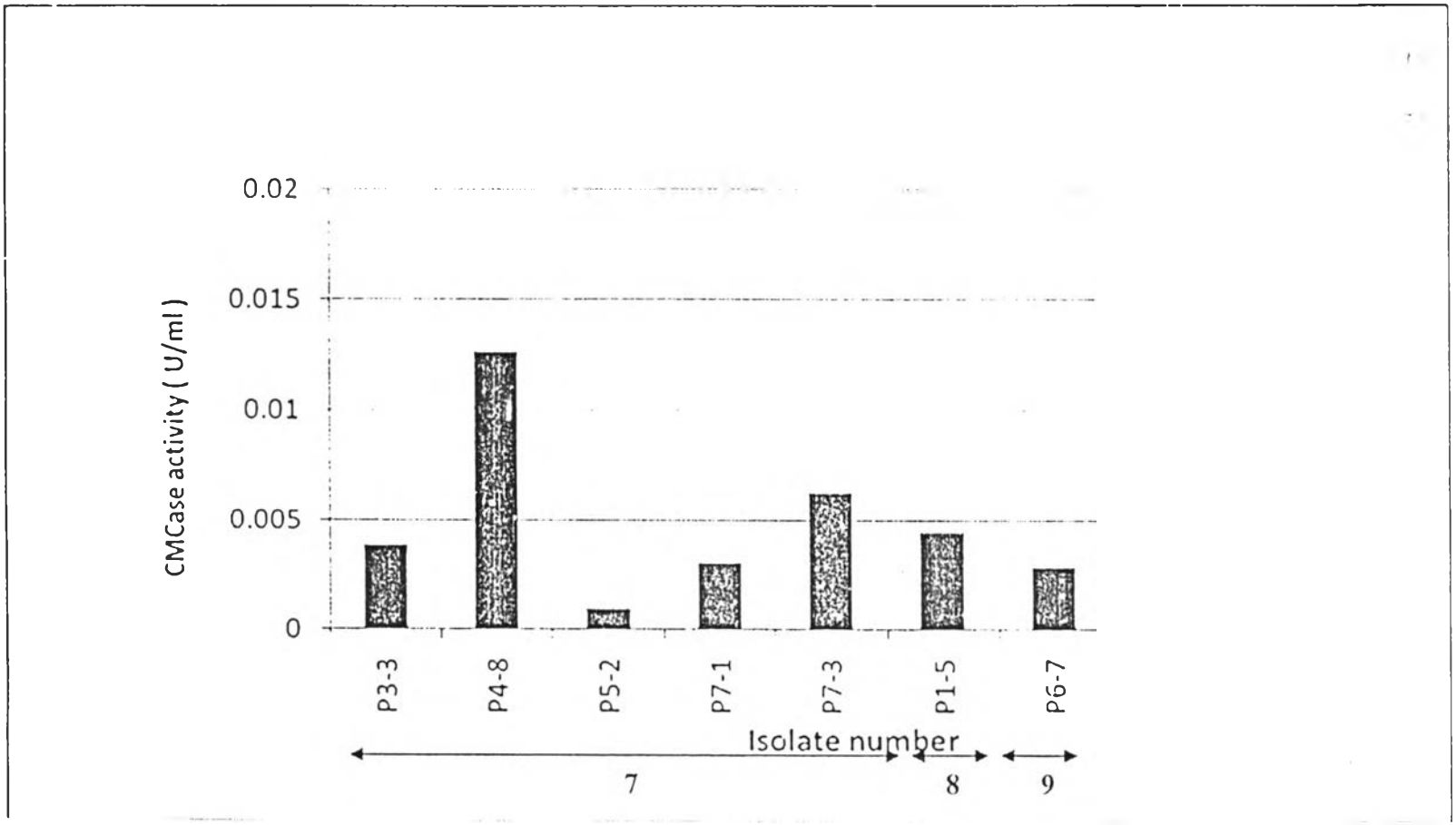


Fig 4.5 Cellulase production of the isolates in Group 7, 8 and 9

2.2 Physiological and biochemical characteristics

All 75 isolates were catalase and oxidase positive. All isolates grew at pH 7-9. Most of the isolates grew at 15, 20, 45 and 50 ^{o} C. 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 4.4). Most of the isolates produced acids from D-cellobiose, D-maltose, D-manitol, D-melibiose, D-melezitose, raffinose, salicin and sucrose. All did not produce acids from gluconate and L-sorbose (Table 4.5 and Appendix D).

Table 4.4 Physiological and biochemical characteristics of the isolates in 9 Groups

Characteristics	Gr.1 2 isolates	Gr.2 9 isolates	Gr.3 11 isolates	Gr.4 13 isolates	Gr.5 12 isolates	Gr.6 21 isolates	Gr.7 5 isolates	Gr.8 1 isolates	Gr.9 1 isolates
Growth with 5% NaCl	-	+	+	+	+(-4)	+ (-1)	+	+	+
Growth at pH 5.0 -8.0	+	+	+	+	+	+	+	+	+
pH 9	-	+	+	+	+	+	+	+	+
Growth at 10 °C	-	-	-	-	-	-	-	-	-
15 °C	-	-(+2)	- (+3)	-	+	+(-4)	+ (-1)	-	+
20 °C -	+	+	+	+	+	+	+	+	+
50 °C	+	+(-1)	+(- 2)	+	-(+1)	+(- 1)	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Anaerobic growth	+(-1)	- (+3)	- (+3)	-(+3)	+(-1)	- (+9)	- (+2)	+	+
Methyl red	-	-	-	-	+	-(+1)	-(+1)	-	+
Voges-Proskauer	-	+(-2)	+(-1)	+(-5)	- (+5)	- (+9)	+(-1)	+	+

Table 4.4 (Cont) Physiological and biochemical characteristics of the isolates.

Characteristics	Gr.1 2 isolates	Gr.2 9 isolates	Gr.3 11 isolates	Gr.4 13 isolates	Gr.5 12 isolates	Gr.6 21 isolates	Gr.7 5 isolates	Gr.8 1 isolates	Gr.9 1 isolates
DNAase	-	-	+(-2)	+	-	- (+ 7)	+	+	-
Utilization of citrate	-	+ (-4)	- (+ 3)	-(+6)	-	+	+	-	-
TSI	-	+ (- 2)	+	+	+(-2)	+	+ (-1)	+	+
Nitrate reduction reaction	-	- (+ 4)	+ (-2)	+(-14)	-	- (+3)	+	+	+
Aesculin Hyhrolysis of	-	+	+	+	+ (-1)	+	+	+	+
Casein	-	+ (-3)	+ (-2)	+	+ (-1)	+	+	+	+
Gelatin	-	+ (-1)	+ (-2)	+	+(-4)	+	+	+	+
Starch	-	+ (-4)	+ (-2)	+	+(-1)	+	+	+	+
Tween 80	-		-	-	-	- (+2)	- (+2)	-	-

Table 4.5 Acid from carbohydrates of isolates in 9 groups

Acid from	Gr.1 2 isolates	Gr.2 9 isolates	Gr.3 11 isolates	Gr.4 13 isolates	Gr.5 12 isolates	Gr.6 21 isolates	Gr.7 5 isolates	Gr.8 1 isolates	Gr.9 1 isolates
D-Amygdalin	+	+ (-1)	-	-	+ (-3)	-	-	-	-
L-Arabinose	-	+ (-1)	(+2)	-	-	-	-	-	-
D-Cellubiose	-	+	+	+ (-4)	+ (-1)	(+9)	-	-	-
D-Galactose	-	+ (-2)	-(+2)	-	-	-	-	-	-
Lactose	+	+ (-2)	(+2)	-	-	-	-	-	-
Raffinose	-	+ (-4)	-	(+2)	-	-	-	-	-
L-Ribose	-	(+1)	-	-	-(+5)	-	-	-	-
Salicin	-	+ (-3)	+ (-4)	+ (-5)	-	+ (-4)	+	+	+
D-Trehalose	-(+1)	+	+ (-3)	-(+3)	-(+6)	+	+(-1)	+	-
D-Xylose	+(-1)	+ (-2)	- (+5)	-	-	- (+2)	-	-	-

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

2.3 Chemotaxonomic characteristics

The representative strains of each 9 different groups were selected and their chemotaxonomic characteristics were determined. All tested strains P5-5 in Group1, P2-1 in Group 2, S10-4 in Group 3, P2-3 and P4-7 in Group 4, S9-2 in Group 5, P6-8 in Group6, P4-8, P5-2, P7-1 and P7-3 in Group 7, P1-5 in Group 8, and P6-7 in Group 9 contained *meso*-diaminopimelic acid as a diagnostic diamino in the cell wall peptidoglycan (Fig 4.6). The predominant menaquinone (MK-7) was found in strains P5-5 in Group1, P2-1 in Group 2, S10-4 in Group 3, P2-3 and P4-7 in Group 4, S9-2 in Group 5, P6-8 in Group6, P4-8, P5-2, P7-1 and P7-3 in Group 7, P1-5 in Group 8, and P6-7 in Group 9. The DNA G+C contents of the tested strains ranged from 41.5-54.2 mol% as shown in Table 4.6

Table 4.6 DNA G+C contents of the representative strains in 9 Groups

Group/ isolate no.	G+C content (mol%)
1. P5-5	54.2
2. P2-1	52.7
3. S10-4	53.5
4. P2-3,	46.3,
P4-7	47.8
5. S9-2	46.5
6. P6-8	42.4
7. P4-8 ,	42.3,
P5-2	41.6,
P7-1,	44. 6,
P7-3	43.2
8. P1-5	41.5
9. P6-7	42.7

On the basis of their phenotypic and chemotaxonomic characteristics, 2 isolates in Group 1 were closed to *Brevibacillus* (Shida *et al.*, 1996). Twenty isolates in Group 2 and 3 showed characteristics that closed to *Paenibacillus* (Ash *et al.*, 1991; 1993), and 53 isolates in Group 4, 5, 6, 7, 8, and 9 were closed to *Bacillus* (Turnbul, 1996 ; Takeuchi and Hatano, 1998 ; Venkateswaran *et al.*, 2003).

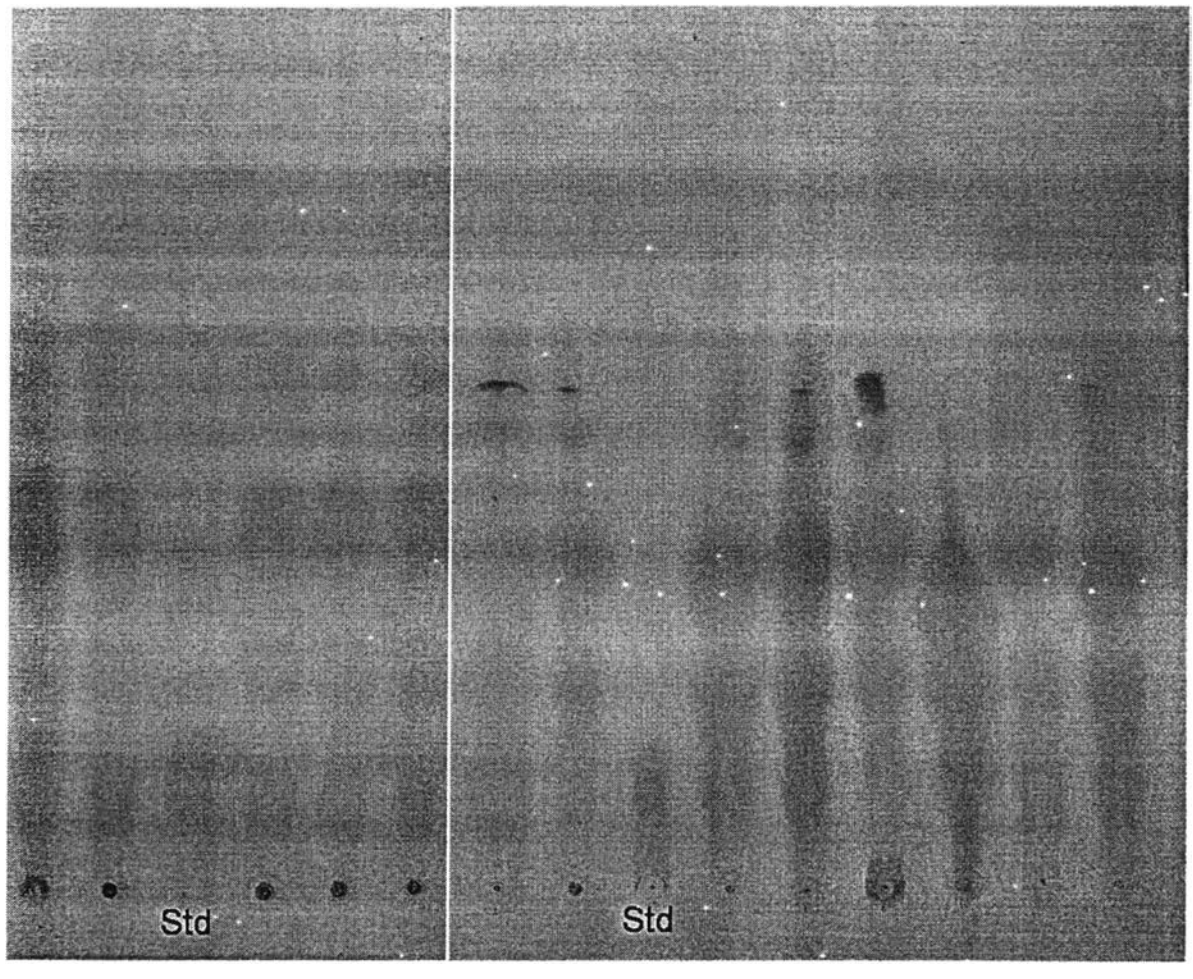


Fig 4.6 Thin Layer Chromatograph of diaminopimelic acid in cell wall of representative strains of each 9 different groups (1, P5-5; 2, P2-1; 3, S10-4; 4, P2-3; 5, P4-7; 6, S9-2; 7, P6-8; 8, P4-8; 9, P5-2; 10, P7-1; 11, P7-3; 12, P1-5; 13, P6-7)

2.4 16S rDNA sequence and phylogenetic tree analysis

The representative strain P5-5 in Group 1 showed 97.2 % similarity to *Brevibacillus agri* DSM 6348^T. Strain P2-1 in Group 2 and S10-4 in Group 3 showed 96.1 and 99.1 % similarities to *Paenibacillus cineris* KCTC 3998^T, respectively (Fig 4.7, Table 4.7), Strains P2-3 and P4-7 in Group 4; S9-2 in Group 5; P6-8 in Group 6; P4-8, P5-2, P7-1, and P7-3 in Group 7; P1-5 in Group 8; P6-7 in Group 9 showed 95.5, 97.8; 95.6; 94.3; 96.1, 96.8 , 94.5, 98.3; 99.3; and 99.9 % similarities to *Bacillus subtilis* KCTC 3135^T, respectively (Fig 4.8, Table 4.8).

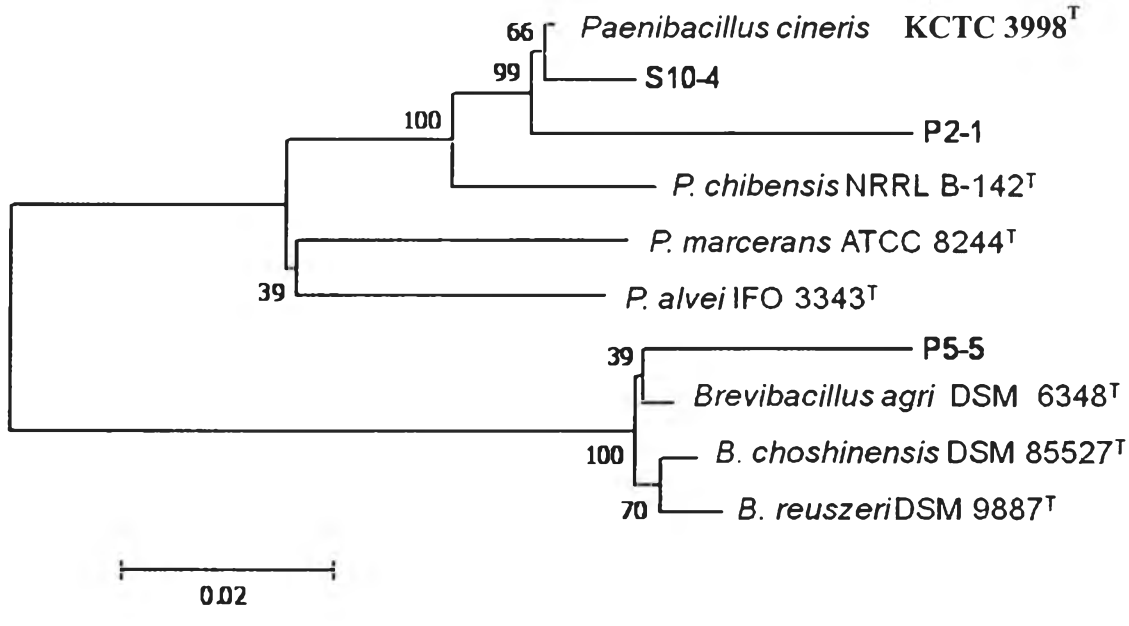


Fig 4.7 Neighbour-joining-tree showing phylogenetic position of strains P2-1, P5-5, S10-4, and related taxa based on 16S rDNA sequences. Bar, 0.02 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications

Table 4.7 Percentage similarities of P2-1, P5-5, S10-4, *Brevibacillus* sp., *Bacillus* spp., and *Paenibacillus* spp.

	1	2	3	4	5	6	7	8	9	10
1.P5-5	100									
2.Brevibacillus agri	97.2	100								
3.Bacillus. choshinensis	96.9	99.2	100							
4.B. treuszeri	96.3	98.8	99.1	100						
5.P. cineris	86.2	88.7	88.3	88.1	100					
6.P. chibensis	85.3	87.9	87.6	87.4	97.3	100				
7.P. macreans	85.7	87.7	87.6	87.4	94.2	93.7	100			
8.P. alvei	86.4	88.1	88	87.6	94.7	93	94	100		
9.P2-1	83.2	85.4	85.1	85.2	96.1	93.6	91	91.2	100	
10.S10-4	85.4	87.8	87.5	87.2	99.1	96.4	93.2	93.8	95.5	100

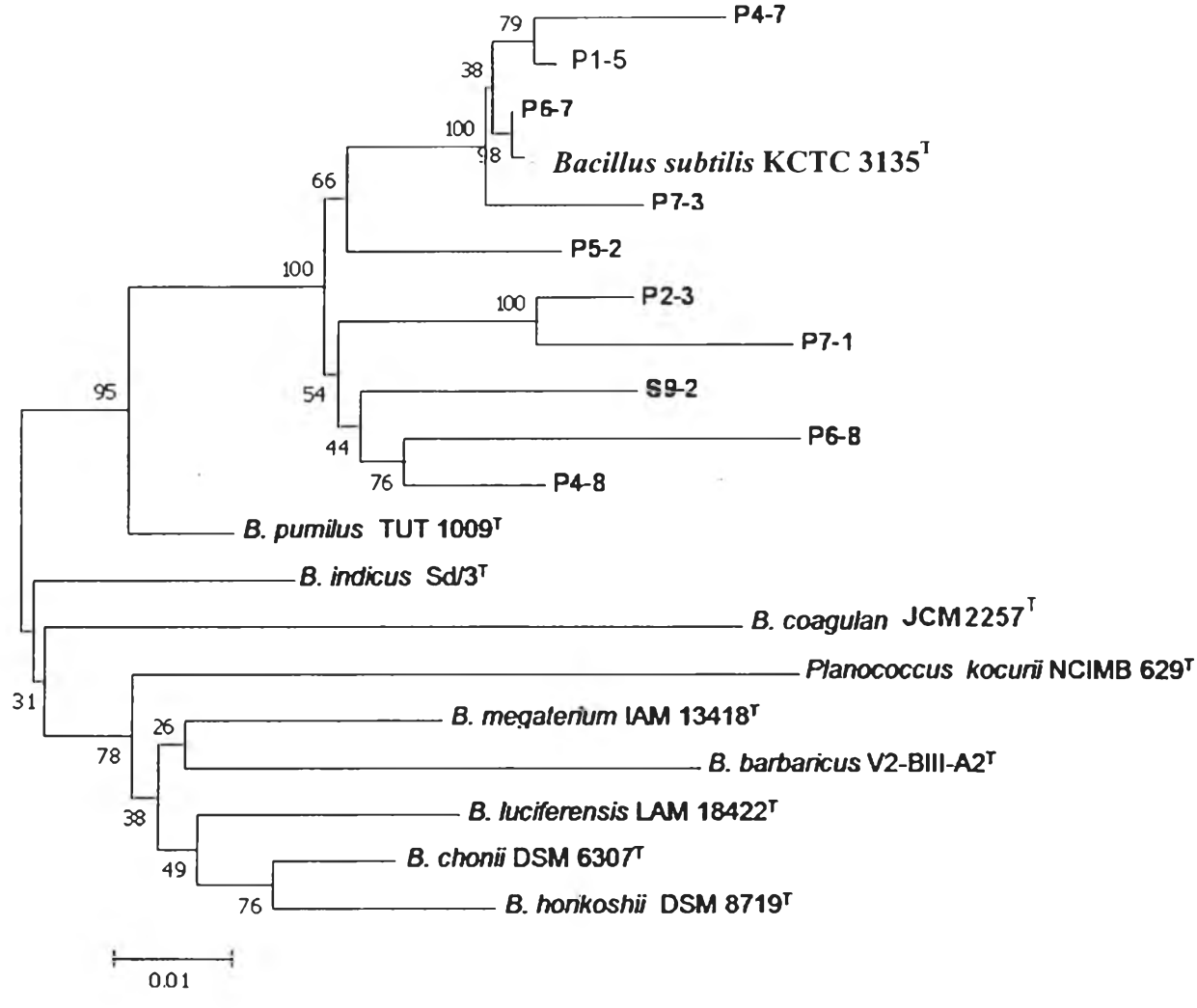


Fig 4.8 Neighbour-joining-tree showing phylogenetic position of strains P1-5, P2-3, P4-7, P4-8, P5-2, P6-7, P6-8, P7-1, P7-3, S9-2, and related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values expressed as percentages of 1000 replications

Table 4.8 Percentage similarities of P1-5, P2-3, P4-7, P4-8, P5-2, P6-7, P6-8, P7-1, P7-3, S9-2 and related *Bacillus* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. P5-2	100																		
2. P4-7	94.8	100																	
3. P7-3	95.8	96.8	100																
4. P6-8	94.3	92.7	93.1	100															
5. P4-8	95.9	95.2	95	95.5	100														
6. P1-5	96.2	98.2	98	94	96.7	100													
7. P2-3	95	94.6	94.4	92.8	96	96	100												
8. P7-1	94	92.4	93.5	92	94.6	94.1	97	100											
9. P6-7	96.9	97.8	98.4	94.5	96.2	99.4	95.6	94.6	100										
10. S9-2	95.4	93.8	94.7	94	96	95.2	94.9	93.9	95.7	100									
11. *B. subtilis*	96.8	97.8	98.3	94.3	96.1	99.3	95.5	94.5	99.9	95.6	100								
12. *B. indicus*	93.1	91.6	92.1	91.3	93.3	93.4	92.6	91.1	93.4	93	93.3	100							
13. *B. cohnii*	92.3	90.9	91.4	89.8	92.4	92.6	91.4	89.9	92.7	91.3	92.6	95.7	100						
14. *B. hurikoshii*	91.4	89.9	90.7	88.7	91.7	91.8	90.5	88.8	91.7	90.5	91.6	94.5	97.1	100					
15. *B. luciferensis*	92.3	90.6	91.3	89.6	92.3	92.4	91.1	89.5	92.5	91.5	92.4	93.6	95.8	95.6	100				
16. *B. pumilus*	95.6	94.1	94.6	93.3	95.7	95.8	94.7	93.2	95.9	95	95.8	96.2	95.7	94.6	94.6	100			
17. *B. megaterium*	92.3	90.3	91.4	89.9	92	92	91.2	90	92.5	91.3	92.4	94.2	96.1	94.7	94.1	94.8	100		
18. *B. coagulans*	89.4	88	88.6	87.2	89.9	89.9	88.8	87.2	89.9	88.9	89.7	91.8	91.1	89.9	89.5	91.3	90.7	100	
19. *B. barbaricus*	90	87.9	88.9	87.2	89.6	89.8	88.6	87.4	90.2	89.2	90.1	92	94	93.1	93	93.1	93.5	88.4	100

Characterization of the isolates

Group 1 contained 2 isolates P5-5 and P6-5. They were spore forming, Gram-positive rods. Colonies were circular, raised, entire margins, smooth, translucent, white colour. Catalase and oxidase positive. They could grow at pH 5.0-8.0 and at 20-50°C but not in 5% NaCl. Negative for MR-VP reaction, DNase, citrate utilization, nitrate reduction, hydrolysis of aesculin, casein, gelation, starch, and Tween 80 (Tables 4.4). P5-5 contained 54.2 mol% of DNA G+C content. P5-5 showed 97.2 % sequence similarity to *Brevibacillus agri* DSM 6348^T. They were identified as *Brevibacillus* (Shida *et al.*, 1996) and differentiated from *Brevibacillus agri* as shown in Table 4.9.

Table 4.9 Differential characteristics of P5-5 in Group 1 and *Brevibacillus agri*

Characteristics	**P5-5**	***Brevibacillus agri***
Anaerobic growth	+	-
Catalase	+	+
Voges-Proskauer reaction	-	+
Growth 10% NaCl	-	-
G+C content (mol%)	54.2	46-57

Group 2 contained 9 isolates and Group 3 had 11 isolates. They were spore forming Gram-positive rods. Colonies were circular, raised, entire margins, smooth, dull, white cream colour. The isolates in these 2 Groups showed similar characteristics however they could be differentiated from each other as shown in Table 4.4. The representative strain P2-1 in Group 2 contained 52.7 mol% of DNA G+C content and showed 96.1% sequence similarity to *Paenibacillus cineris* KCTC 3998^T (Table 4.10).
The representative strain S10-4 in Group 3 contained 53.5 mol% of DNA G+C content and showed 99.1 % sequence similarity to *Paenibacillus cineris* KCTC 3998^T (Table 4.10). This strain should be identified as *Paenibacillus cineris* (Logan *et al.*, 2004)

Table 4.10 Differential characteristics of isolates in Group 2, 3 and *P. cineris* KCTC 3998^T

Characteristics	P2-1	S10-4	*P. cineris* KCTC 3998^T
Gram staining	+	+	-
Oxidase	+	+	+
Catalase	+	+	+
Voges Proskauer test	+	-	-
Growth at/in			
3% (w/v) NaCl	+	+	+
5°C	-	-	-
10°C	-	-	-
37°C	+	+	+
pH 5.6	+	+	+
Hydrolysis of			
Casein	+	+	+
Starch	-	-	ND
Gelatin	+	-	-
Esculin	+	+	+
Utilization of			
D-Galactose	+	+	ND
D-Xylose	+	+	ND
Acid production from			
D-Mannitol	-	-	+
D-Sorbitol	-	-	+
D-Sucrose	+	+	+
D-Melibiose	+	+	+
Amygdalin	+	W	+
L-Arabinose	+	+	+
DNA G+C (mol%)	**52.7**	**53.5**	**51.5**

Group 4 to Group 9 contained 53 isolates. All were Gram–positive bacilli, catalase and oxidase positive. Group 4 showed circular/ irregular, raised, entire undulate margins, smooth, dull, white colour. Colonies were circular, flat, undulate margins, smooth, dull, white colour in Group 5. Group 6 isolates showed circular, raised, entire margins, smooth, dull, viscid, white colour colonies. Isolates in Group 7 and 8 showed irregular, raised, lobate margins, rough, dull, yellow and yellowish white colour colonies. An isolate in Group 9 was circular, raised, undulate margins , wrinkled, dull, white colour colonies (Table 4.2). The representative strains, P2-3 and P4-7 in Group 4 grew in anaerobic condition but not at 5% NaCl. No acid production from L-arabinose, raffinose, salicin, lactose, and D-xylose. P2-3 showed positive for gelatin hydrolysis but negative for VP reaction and citrate. P4-7 showed negative for gelatin hydrolysis and VP reaction. P2-3 and P4-7 contained 46.34 and 47.84 mol % of DNA G+C contents and showed 95.5 and 97.8 % sequence similarity to *Bacillus subtilis* KCTC 3135^T, respectively (Table 4.11).

Strain S9-2 in Group **5** grew in anaerobic condition but not at 50° C. Negative for citrate utilization and nitrate reduction. No acid production from L-arabinose, raffinose, lactose and D-xylose. This strain contained 46.51 mol % of DNA G+C content and showed 95.6 % sequence similarity to *Bacillus subtilis* KCTC 3135^T(Table 4.11).

P6-8 strain in Group 6 showed negative for Voges-Proskauer reaction and nitrate reduction. No acid production from L-arabinose, raffinose, lactose and D-xylose. P6-8 showed the DNA G+C contents 42.37 mol % and showed 94.3 % sequence similarity to *Bacillus subtilis* KCTC 3135^T (Table 4.11).

Table 4.11 Differential characteristics of isolate in Group 4, 5, 6 and *B.subtilis* KCTC 3135^T

Characteristics	**P2-3**	**P4-7**	**S9-2**	**P6-8**	**P4-8**	***B. subtilis* KCTC 3135^T**
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Growth at 50° C	+	+	-	+	+	+
at pH 6.0	+	+	+	+	+	+
Growth with 5% NaCl	-	-	+	+	+	+
Anaerobic growth	+	+	+	-	+	-
Hyhrolysis of starch	+	+	+	+	+	+
casein	+	+	+	+	+	+
Gelatin liquefaction	+	-	+	+	+	+
Utilization of citrate	-	+	-	+	+	+
Nitrate reduction	+	+	-	-	+	+
Voges-Proskauer reaction	-	-	+	-	+	+
Acid from L-Arabinose	-	-	-	-	-	+
Raffinose	-	-	-	-	-	+
Salicin	-	-	+	+	+	+
Galactose	-	-	-	-	-	-
Lactose	-	-	-	-	-	+
D-Xylose	-	-	-	-	-	+
DNA G+C (mol%)	46.3	47.8	46.5	42.4	42.3	43.0

The representative strains in Group 7, P4-8, P5-2, P7-1, and P7-3 did not produce acid from L-arabinose, raffinose, lactose and D-xylose. P4-8 and P7-3 grew in anaerobic condition. P4-8, P5-2, P7-1 and P7-3 contained 42.3, 41.6, 44. 6 , and 43.2 mol % of DNA G+C contents and showed 96.1, 96.8, 94.5 and 98.3% sequence similarities to *Bacillus subtilis* KCTC 3135^{T}, respectively (Table 4.12).

The representative strain in Group 8, P1-5 grew in anaerobic condition. Negative for citrate. No acid production from L-arabinose, raffinose, lactose and D-xylose. P1-5 contained 41.5 mol% of DNA G+C content and showed 99.3 % sequence similarity to *Bacillus subtilis* KCTC 3135^{T}(Table 4.12).

The representative strain in Group 9, P6-7 grew in anaerobic condition. Negative for citrate. No acid production from L-arabinose, raffinose, lactose, and D-xylose. P6-7 showed 42.7 mol% of DNA G+C content and showed 99.9 % sequence similarity to *Bacillus subtilis* KCTC 3135^{T} (Table 4.12). This strain should be identified as *Bacillus subtilis* (Shida *et al.*, 1997).

As mentioned above, the cellulase producing bacteria were isolates and found to be diverse species in soil samples collected in Nan province. One strain of *Brevibacillus*, 1 *Paenibacillus*, and 7 *Bacillus* strains isolated from Pua district and one strain of *Bacillus* from Santisuk district were the novel species. One *Paenibacillus* strain should be identified as *P. cineris* from Santisuk district and 2 *Bacillus* strains from Pua district should be identified as *Bacillus subtilis*. Their distribution and identification were shown in Table 4. 14. However, the strains that showed the 16S rDNA sequence similarity over 97% should be done for the DNA-DNA hybridization experiment to confirmed their taxonomic status.

Table 4.12 Differential characteristics of isolate in Group 7, 8, 9 and *B.subtilis* KCTC 3135^T

Characteristics	P5-2	P7-1	P7-3	P1-5	P6-7	*B. subtilis* KCTC 3135^T
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Growth at 50° C	+	+	+	+	+	+
at pH 6.0	+	+	+	+	+	+
Growth with 5% NaCl	+	+	+	+	+	+
Anaerobic growth	-	-	+	+	+	-
Hyhrolysis of starch	+	+	+	+	+	+
casein	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+
Utilization of citrate	+	+	+	-	-	+
Nitrate reduction	+	+	+	+	+	+
Voges-Proskauer reaction	+	+	+	+	+	+
Acid from L-Arabinose	-	-	-	-	-	+
Raffinose	-	-	-	-	-	+
Salicin	+	+	+	+	+	+
Galactose	-	-	-	-	-	-
Lactose	-	-	-	-	-	+
D-Xylose	-	-	-	-	-	+
DNA G+C (mol%)	41.6	44.6	43.2	41.5	42.7	43.0

Table 4.14 Distribution and identification of the representative strains

District	Group	Isolate no.	% Similarity	Identification
Pua	1	P5-5	97.2	*Brevibacillus* sp. nov
	2	P2-1	96.1	*Paenibacillus* sp. nov
Santisuk	3	S10-4	99.1	*Paenibacillus* sp.
Pua	4	P2-3, P4-7	95.5, 97.8	*Bacillus* sp. nov
Santisuk	5	S9-2	95.6	*Bacillus* sp. nov
Pua	6	P6-8	94.3	*Bacillus* sp. nov
	7	P4-8, P5-2, P7-1, P7-3	96.1, 96.8, 94.5, 98.3	*Bacillus* sp. nov *Bacillus* sp. nov
	8	P1-5	99.3	*Bacillus* sp.
	9	P6-7	99.9	*Bacillus* sp.

3. Effect of pH and temperature on cellulase production

Strain P4-6 in Group 4, P3-1 in Group 6, and P4-8 in Group 7, selected as the most highest cellulase producing strains and the representatives of high hydrolysis capacity (HC) value strains, P2-1 in Group 2, P7-7 in Group 3, and P2-3 in Group 4 were determined on the various pH and temperature effects.

Strain P2-1, P2-3, P3-1, P4-6, P4-8, and P7-7 produced maximum cellulase at pH 7.0 (Fig. 4.9) and at 50° C (Fig. 4.10).

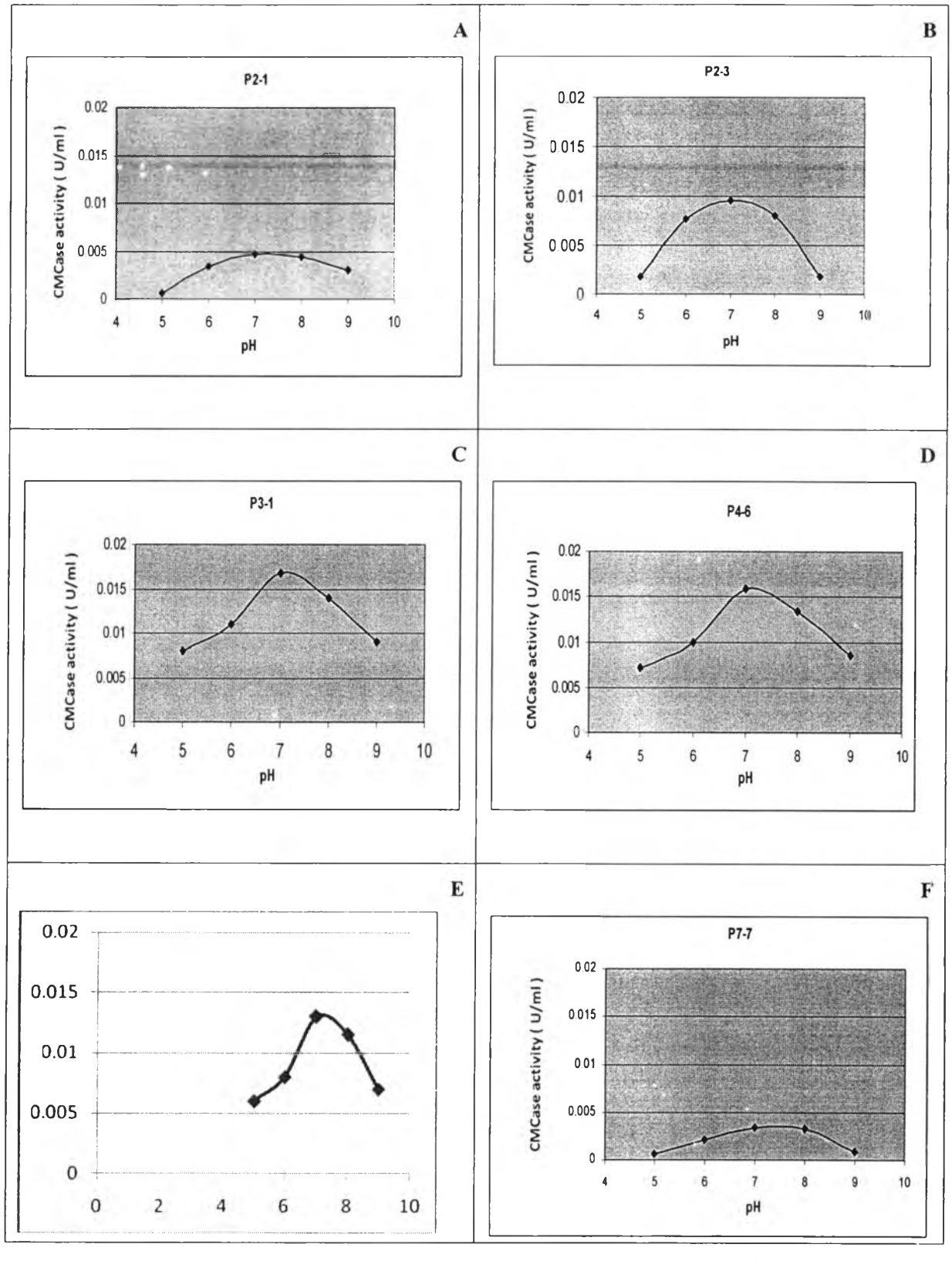


Fig 4.9 Effect of pH on cellulase production of strains P2-1, P2-3, P3-1, P4-6, P4-8 and P7-7 (A-F).

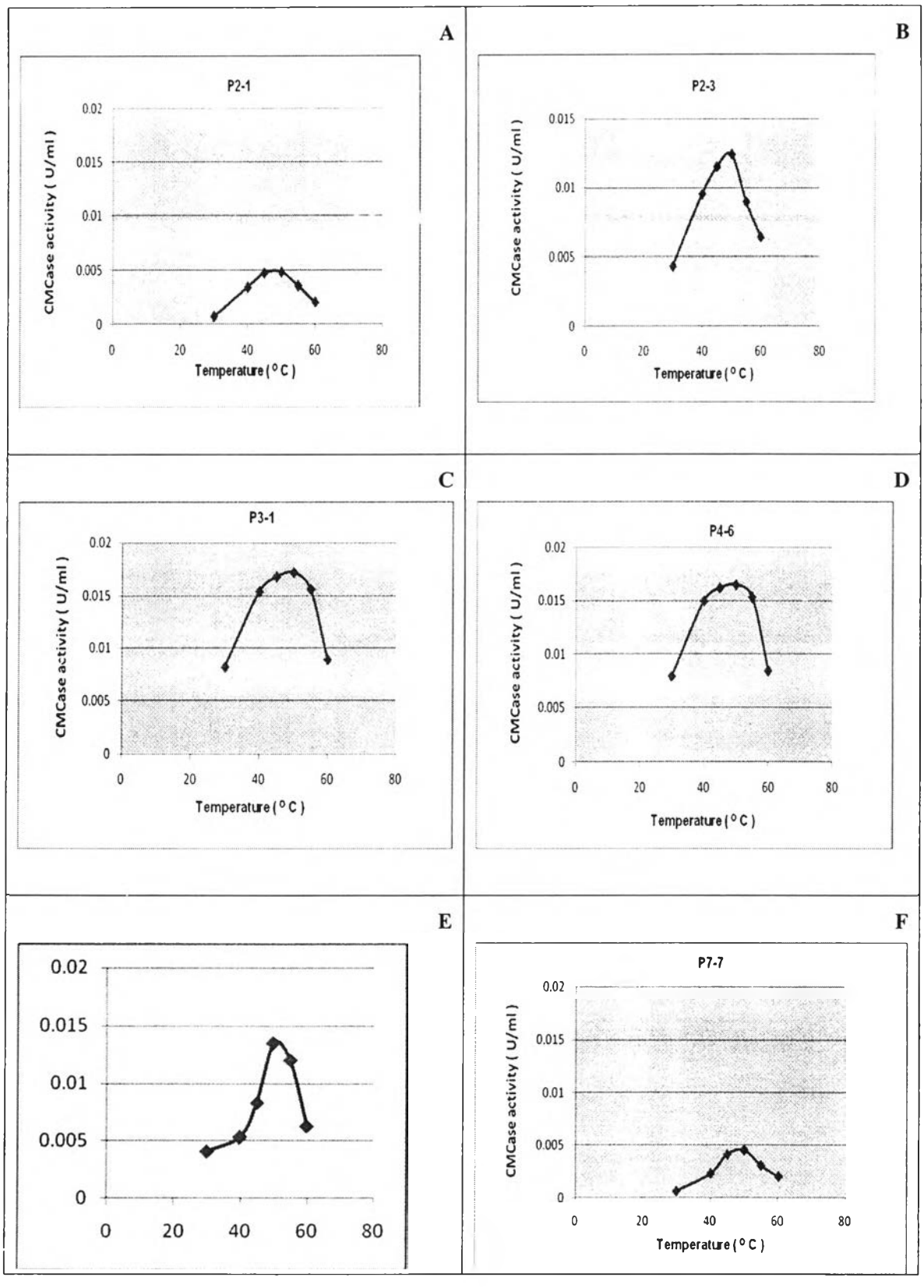
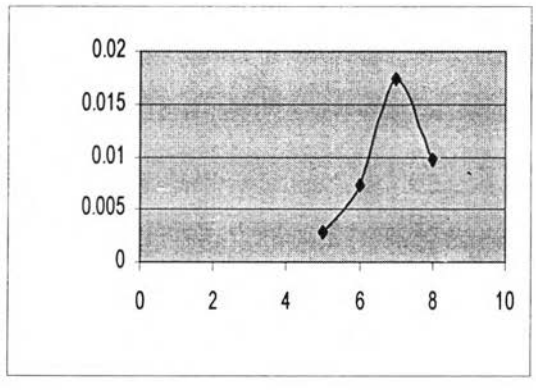


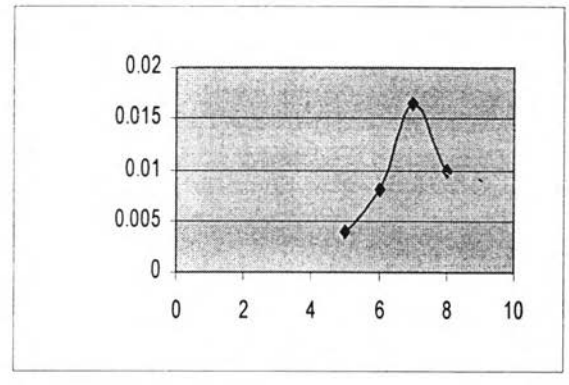
Fig 4.10 Effect of temperature on cellulase production of strains P2-1, P2-3, P3-1, P4-6, P4-8 and P7-7 (A–F).

4. Effect of pH and temperature on cellulase activity

The most highest cellulase produicng strains P3-1 and P4-6 grown in CMC medium at various pH and temperature were selected to determined for their activities on different pH and temperature.

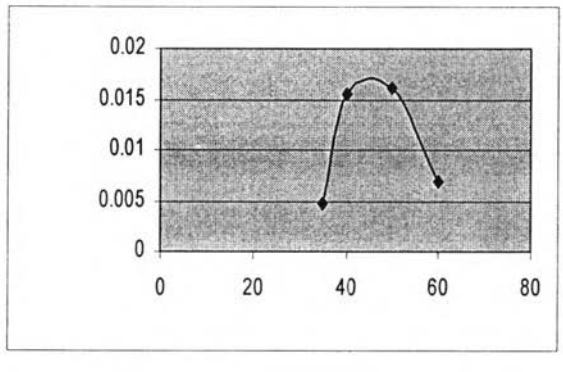
Optimum pH and temperature for cellulase activity of P3-1 and P4-6 were at pH 7.0 (Fig. 4.11) and at 50° C (Fig. 4.12).

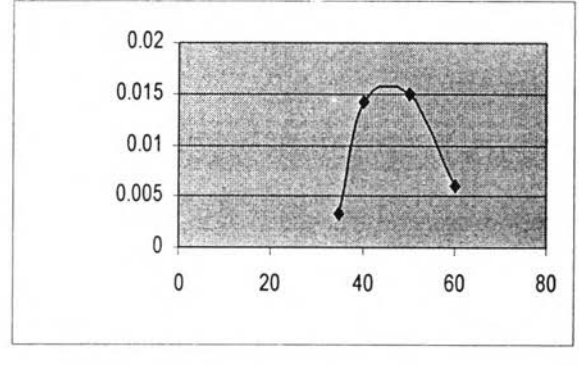




A B

Fig 4.11 Optimum pH for cellulase of P3-1 and P4-6





A B

Fig 4.12 Optimum temperature for cellulase of P3-1 and P4-6