

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Isolation and Screening of Lactic Acid Bacteria.

The objective of this experiment was to select the strains of human origin that have acid and bile tolerant. So, the microorganisms that isolated from healthy infant feces would be more likely to colonize in the human intestine. The dilutions of 55 fecal samples were spread on MRS agar plus bromocresol purple as indicator. After 24 hours of incubation, Lactic Acid Bacteria grew on the media. There were 79 isolates of 55 fecal samples grew as a yellow colony on MRS agar with addition of bromocresol purple (0.04 g/l) as indicator (Fig.4.1). The number of lactic acid bacteria in the infant feces ranged between 2.7×10^6 to 3.4×10^9 CFU/ml and appropriate dilution ranged between 5 - 7. Only 64 of 79 isolates were lactic acid bacteria that showed the ability to hydrolyzed CaCO_3 (Fig.4.2).



Figure 4.1 The yellow colony of LAB on MRS agar plus bromocresol purple as indicator after incubated at 37°C for 24 hours.



Figure 4.2 Acid production clear zone of LAB on MRS agar plus CaCO₃

On figure 4.2, only lactic acid bacteria showed the clear zone of acid production because the lactic acid degraded calcium carbonated around. There are 3 isolates of LAB showed acid production clear zone, but some isolates were not the clear zone because they didn't show acid production. Similarity, Chen *et al.* (2006) distinguished the acid-production bacteria from other bacteria, 1% CaCO₃ was added to the MRS agar and only colonies with a clear zone around colony.

Table 4.1 Clear zone of acid production by 64 isolates of lactic acid bacteria

Number	Isolate	Clear zone of acid production (mm.)	Number	Isolate	Clear zone of acid production (mm.)
1	F21	15.0	26	F14/2	8.0
2	F33/2	14.0	27	S3/2	8.0
3	F28/2	13.0	28	S2/2	7.5
4	F40/1	13.0	29	F14/1	7.0
5	F26	12.8	30	F20	7.0
6	F25/2	12.0	31	F34	7.0
7	F27	12.0	32	F38	7.0
8	F33/1	12.0	33	S4/1	7.0
9	F35/2	12.0	34	S7/2	7.0
10	F39	12.0	35	S2/4	6.5
11	F35/1	11.5	36	F7	6.0
12	F14/3	11.0	37	F23/1	6.0
13	F32	11.0	38	F24	6.0
14	F4	10.0	39	F28/1	6.0
15	F9	10.0	40	F31	6.0
16	F16/1	10.0	41	S51/2	6.0
17	F1/1	9.0	42	S6	6.0
18	F1/2	9.0	43	S3/3	6.0
19	F5	9.0	44	S5	6.0
20	S8	9.0	45	S55/1	6.0
21	S2/1	9.0	46	S56/1	6.0
22	S1	9.0	47	S56/2	6.0
23	S2/3	8.5	48	F11	5.5
24	F22	8.2	49	S7	5.5
25	F6	8.0	50	F10/1	5.0

Table 4.1 (Continue)

Number	Isolate	Clear zone of acid production (mm.)	Number	Isolate	Clear zone of acid production (mm.)
51	F10/2	5.0	58	F12	4.0
52	F17	5.0	59	S51/1	4.0
53	S52/1	5.0	60	S4/3	4.0
54	S7/2	5.0	61	S54/3	4.0
55	S4/2	5.0	62	S52/2	3.0
56	S53/2	5.0	63	S53/1	3.0
57	F18	4.2	64	S54/1	3.0

In table 4.1, sixty-four of 79 LAB isolates appeared as yellow colony on MRS agar plus bromocresol produced clear zone of acid production. The average diameter of clear zone were 7.64 mm. These 64 isolates were selected from MRS agar plates and purified by re-plating on MRS agar plates.

4.2 Selection of Acid-tolerant Lactic Acid Bacteria.

Sixty-four lactic acid bacteria were tested for their ability to tolerate low pH. Survival of 64 isolates was examined in MRS broth that adjusted pH value at 2, 3 and 4. After incubated at 37°C, turbidity were observed in comparison with a control (MRS broth pH 6.2). The result indicated that 39 of 64 isolates were acid-tolerant LAB that survived at pH 2, 3 and 4 (Table 4.2). There were 11 isolates namely, F9, F21, F20, F16, F26, F28/2, F35/2, F14/3, F40, F6 and S7 survived at pH 4. Whereas, isolate F14/1, F10/2, S2/2, F32/2, F27, F33/2, F4, F7, F23 and S51/2, survived at pH 3. At pH 2, there were 18 isolates namely, F25, F32, F5, F22, F14/2, F1/2, S2/1, S3/3, F31, S1, S5, S6, F17, S8, F38, S6/3, S7/2 and S8/1 survived at this condition.

Table 4.2 Eleven isolates of lactic acid bacteria that grew at pH 4.

Number	Isolate	Number of Cells (x 10 ⁸ CFU/ml)
1	F9	1.49
2	F28/2	1.39
3	S7	1.32
4	F6	1.23
5	F26	1.21
6	F21	1.20
7	F35/2	0.97
8	F20	0.95
9	F40	0.88
10	F16	0.87
11	F14/3	0.61

Table 4.3 Ten isolates of lactic acid bacteria that grew at pH 4 and 3.

Number	Isolate	Number of Cells (x 10 ⁸ CFU/ml)
1	F27	1.40
2	F4	1.11
3	F7	1.08
4	F14/1	1.01
5	F32/1	0.99
6	F33/2	0.83
7	F23	0.78
8	F10/2	0.65
9	S2/2	0.63
10	F51/2	0.45

Table 4.4 Eighteen isolates of lactic acid bacteria that grew at pH 4, 3 and 2.

Number	Isolate	Number of Cells (x 10 ⁸ CFU/ml)
1	S1	1.28
2	F31	1.20
3	S8	1.12
4	S3/3	1.07
5	F17	1.00
6	S54/3	1.00
7	F5	0.95
8	S6	0.95
9	F32/1	0.93
10	S2/1	0.92
11	F25/2	0.90
12	F14/2	0.89
13	S55/2	0.84
14	S56/1	0.77
15	F22	0.64
16	S5	0.60
17	F1/2	0.51
18	F38	0.30

There were 11, 10 and 18 isolates of 64 lactic acid bacteria survived at pH 4, 3 and 2, respectively (Table 4.2). At pH 4, the number of cells ranged between 61-149 x 10⁶ CFU/ml. At pH 3, the number of cells ranged between 45-140 x 10⁶ CFU/ml and between 30-128 x 10⁶ CFU/ml. for pH 2. It was found that the average number of cells at pH 4 (110.18 x 10⁶ CFU/ml) was higher than other pH. On the other hand, the average number of cells at pH 2 was the lowest about 88.17 x 10⁶ CFU/ml. The pH value had adverse effect to cell viability. Acid condition (lower pH) decreased the number of cells.

The survival of bacteria in gastric juice depends on their ability to tolerate low pH. The pH of excreted HCl in stomach is 0.9. However, the presence of food raises the pH value to pH 3. So, we tested in pH value 2, 3 and 4. Gupta *et al.* (1996) observed that only 2 of 7 *L. acidophilus* strains test exhibited growth at pH 3. Similarly, the results of Suscovic *et al.* (1997) on *L. acidophilus* strain suggested a high acid tolerance at pH 3.0. From the result, it was found that 39 isolates of 64 LAB are acid tolerant lactic acid bacteria. Therefore, it has been assumed that these isolated strains may survive passage through the digestive system that has specific condition such as the low pH of the stomach. All of them were tested for bile salts tolerance in next step.

4.3 Selection of acid and bile tolerant lactic acid bacteria

Bile tolerance was known to be one of essential properties required for LAB to survive in the small intestine and the role it plays in physiological functions (Park *et al.*, 2002). Many authors investigated the effect of acid and bile on survival of LAB. Erkkila and Petaja (2000) reported that strains of *Lactobacillus sake* (RM10) and *Pediococcus acidilactici* (P2) had the best ability under acidic conditions (pH 3) and 0.3% bile salt, the number of bacteria was between 5.1-7.4 log CFU/ml. According to Kim *et al.* (1999) which examined the effect of bile concentration in range of 0-0.4% on the *Lactobacillus lactis* survival, they reported that all bacterial cells were killed at 0.2% and higher. Gilliland *et al.* (1984) considered 0.3% bile salts as a critical concentration to screen for resistant strains. Thus, we selected 0.15% and 0.3% bile salts concentration to tested for tolerant isolates.

Thirty-nine isolates of acid tolerant lactic acid bacteria were tested for their ability to grow in MRS supplemented with 0.15% and 0.30% bile salts. The results indicated that 16 of 39 of acid-tolerant isolates survived in 0.15% and 0.30% bile salts in MRS broth at pH 2 (Table 4.7), 3 (Table 4.6) and 4 (Table 4.5).

Table 4.5 Number of lactic acid bacteria after incubation in 0.15% and 0.3% bile salts in MRS broth at pH 4

pH	Bile salts (%)	Time (h)	Number of lactic acid bacteria (x 10 ⁵ CFU/ml)			
			F9	F16	F20	F40
4	0.15	0	400	302	111	450
		0.5	184	268	80.0	180
		1.0	17.4	8.40	3.50	10.5
		2.5	0.81	0.54	0.13	0.70
		4.0	-	0.05	0.01	0.03
4	0.30	0	525	564	210	327
		0.5	480	237	190	147
		1.0	42.0	13.0	2.10	4.30
		2.5	0.77	0.02	0.09	0.03
		4.0	-	0.01	0.03	0.01

At pH 4, 4 of 11 isolates survived after 4.0 h. in 0.15% and 0.3% bile salts concentration. Resistant isolates showed slightly decrease in viable cell numbers. The number of viable cells were between 0.01–564 x 10⁵ CFU/ml (Fig 4.3 and Fig 4.4). Bile salts had little effect on the viable cells at acidic condition. They were able to survive the bile salt concentration. However, the difference between 0.15% and 0.3% bile salts on the survival capacity of the tested isolates was not clear.

All rights reserved

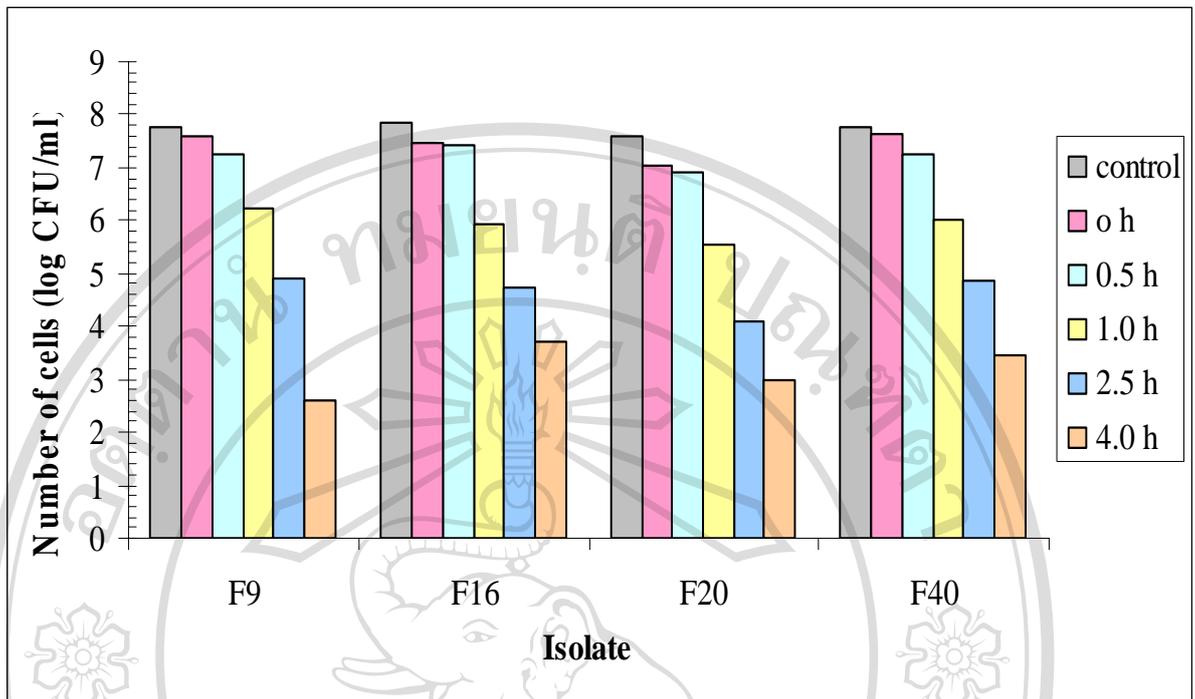


Figure 4.3 Number of lactic acid bacteria at pH 4, after incubation in MRS broth with 0.15% bile salts for 0, 0.5, 1.0, 2.5 and 4.0 h compare with control, MRS broth without bile salts.

The data from table 4.5 were used in Fig. 4.3 and Fig. 4.4. At 0.15% bile salts, the declination of viable bacteria decreased with time. More time more decreasing of bacteria. After 1 h of incubation, the viable bacteria were between 5.5 – 6.0 log CFU/ml. Then at 2.5 h, the number of bacteria decreased to 4.1 log CFU/ml and at least less than 3.0 log CFU/ml of isolate F9. On the other hand, isolate F16 was the best tolerant at 0.15% bile salts in MRS broth at pH 4.

ลิขสิทธิ์โดย Chiang Mai University
All rights reserved

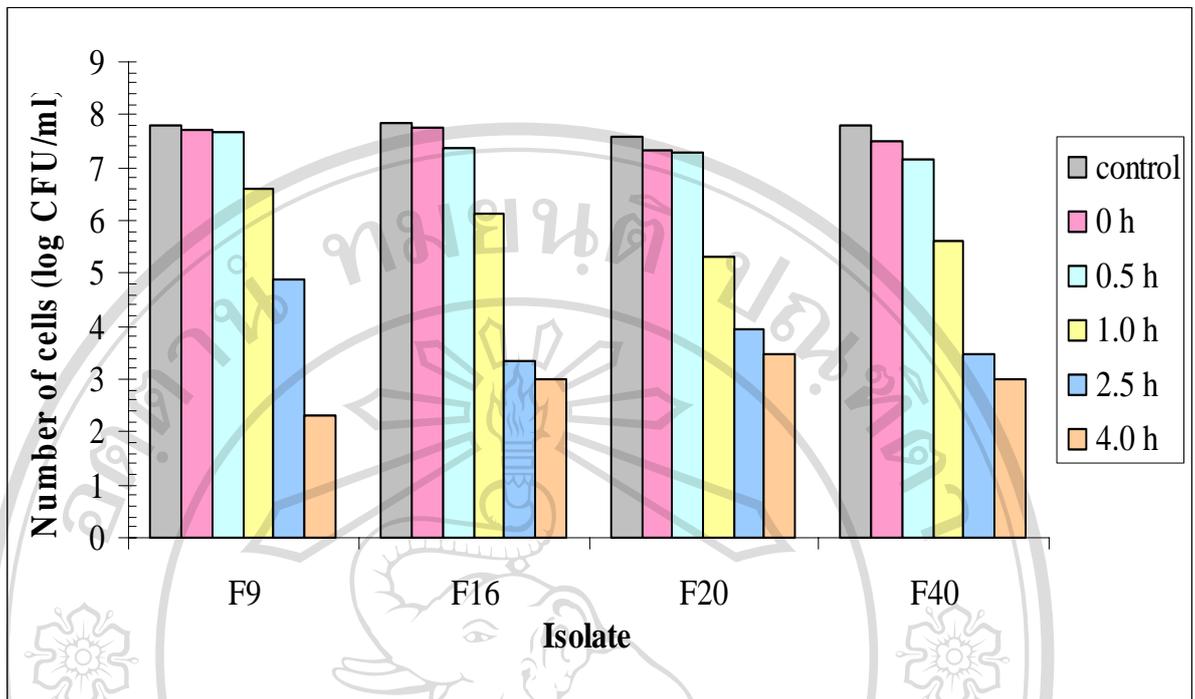


Figure 4.4 Number of lactic acid bacteria at pH 4, after incubation in MRS broth with 0.30% bile salts for 0, 0.5, 1.0, 2.5 and 4.0 h compare with control, MRS broth without bile salts.

At 0.30% bile salts, the declination of viable bacteria decreased to time. Similar to 0.15% bile salts.. After 1 h of incubation , the viable bacteria were between 5.3 – 6.6 log CFU/ml. Then at 2.5 h, the number of bacteria decreased to 3.3 log CFU/ml and at least less than 3.0 log CFU/ml for isolate F9. So, isolate F20 was the best tolerant at 0.30% bile salts in MRS broth at pH 4.

Table 4.6 Number of lactic acid bacteria after incubation in 0.15% and 0.3% bile salts in MRS broth at pH 3

pH	Bile salts (%)	Time (h.)	Number of lactic acid bacteria ($\times 10^5$ CFU/ml)					
			F7	F4	S2/2	F14/1	F10/2	F27
3	0.15	0	128	363	182	600	724	696
		0.5	7.00	39.0	13.0	7.30	0.34	620
		1.0	0.72	0.40	0.02	5.70	0.20	52.0
		2.5	-	-	-	1.90	-	0.58
		4.0	-	-	-	0.79	-	0.12
3	0.3	0	139	320	151	480	51.0	364
		0.5	1.00	16.0	50.0	4.70	0.16	236
		1.0	0.17	0.02	0.138	0.40	0.015	11.3
		2.5	-	-	-	0.170	-	0.34
		4.0	-	-	-	0.140	-	0.07

At pH 3, six of 10 isolates tolerated and survived in 0.15% and 0.3% bile salts concentration. The number of viable cells present between 0.07-724 $\times 10^5$ CFU/ml. The number of bacteria decreased after 0.10 h. of incubation time at 0.15% bile salts concentration as same as 0.30% bile salts concentration. Only isolate F14/1 and F27 had a difference (Figure 4.5 and Figure 4.6), they were more stable than other isolates (6 isolates) at this pH. They grew in both bile salts concentration and survived after 4.0 h. Whatever, the number of bacteria of them were slightly because of the direct effect of higher acidity in medium may decrease the viable cell.

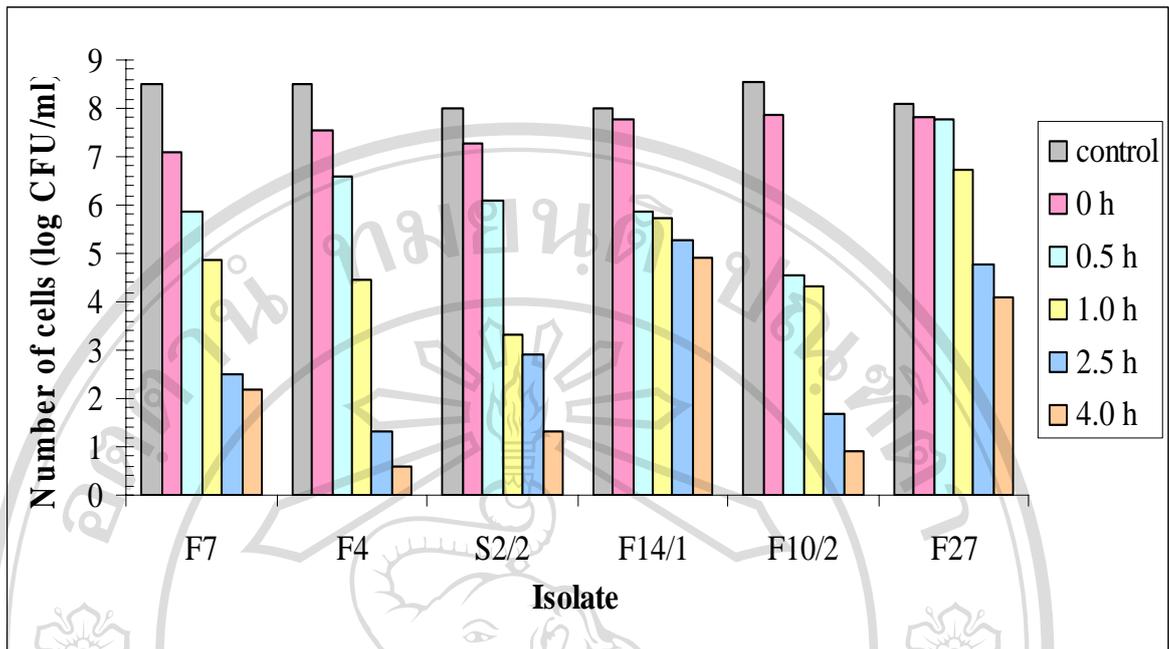


Figure 4.5 Number of lactic acid bacteria at pH 3, after incubation in MRS broth with 0.15% bile salts for 0, 0.5, 1.0, 2.5 and 4.0 h compare with control, MRS broth without bile salts.

The data from table 4.6 were used in Fig. 4.5 and Fig. 4.6. After incubated in 0.15% bile salts at various time, the viable bacteria decreased from 7.8 log CFU/ml (0 h) to 1.0 log CFU/ml. At 1 h of incubation time, the viable bacteria were between 4.5 – 7.7 log CFU/ml. After 2.5 h, the number of bacteria decreased rapidly to less than 3.0 log CFU/ml and nearly to stop the growth at 4.0 h. Exceptional isolate F14/1 and F27, they can survive in 0.15% bile salts and had a number of viable bacteria 4.89 and 4.08 log CFU/ml, respectively.

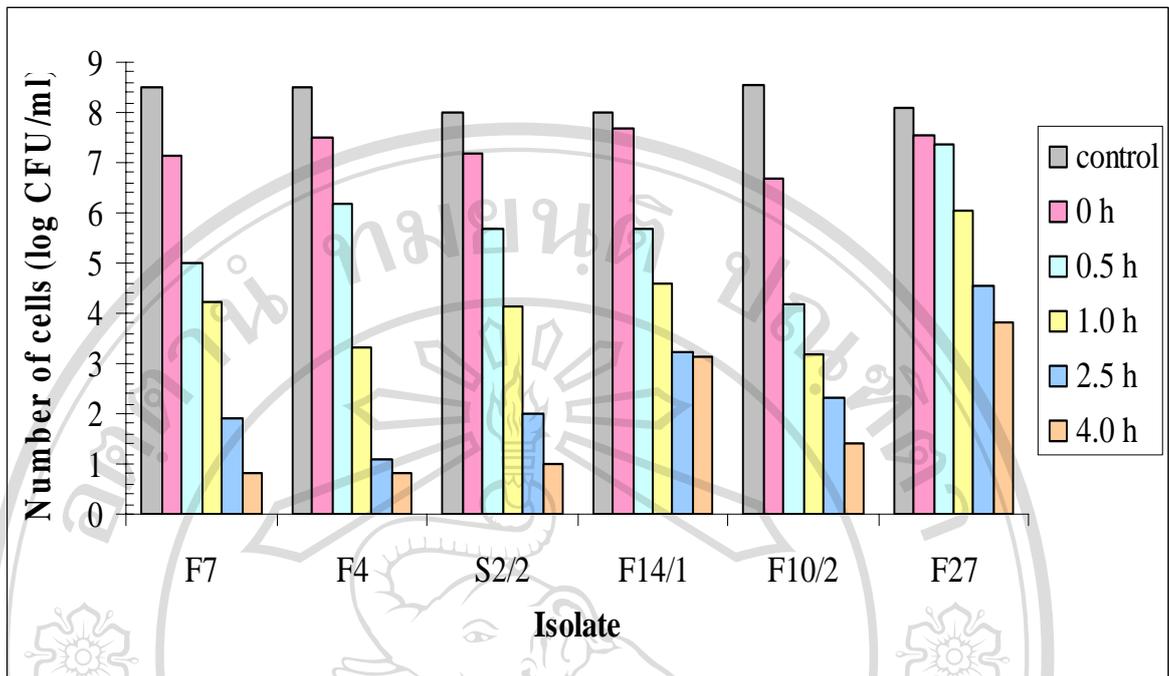


Figure 4.6 Number of lactic acid bacteria at pH 3, after incubation in MRS broth with 0.30% bile salts for 0, 0.5, 1.0, 2.5 and 4.0 h compare with control, MRS broth without bile salts.

After incubated in 0.30% bile salts at various time, the viable bacteria decreased from 7.6 log CFU/ml (0 h) to 1.0 log CFU/ml. At 1 h of incubation time, the viable bacteria were between 3.17 – 6.0 log CFU/ml. Compare with 0.15% bile salts, the rate of bacteria at 0.3% bile salts were less than at 0.15% bile salts. Thus, the result indicated that high concentration of bile salts have a direct effect to the number of viable bacteria. After 2.5 and 4.0 h, the number of bacteria decreased rapidly to less than 3.0 log CFU/ml. Unless two isolates that can tolerate in 0.30% bile salts and had a number of viable bacteria more than 3.0 log CFU/ml.

Table 4.7 Number of lactic acid bacteria after incubation in 0.15% and 0.3% bile salts in MRS broth at pH 2

pH	Bile salts (%)	Time (h.)	Number of lactic acid bacteria ($\times 10^6$ CFU/ml)					
			F5	F14/2	S2/1	S1	S5	F31
2	0.15	0	350	308	535	380	300	602
		0.5	160	200	254	124	60.0	196
		1.0	74.0	180	111	39.0	3.00	113
		2.5	0.20	0.82	2.20	0.100	0.003	73.0
		4.0	0.099	0.11	0.008	0.002	0.002	0.051
2	0.3	0	406	222	483	300	186	594
		0.5	128	66	127	89.0	8.40	159
		1.0	27	0.4	73	31.0	0.159	2.00
		2.5	0.16	0.032	0.03	0.063	0.008	0.016
		4.0	-	-	-	-	-	0.003

The data from table 4.7 were used in Fig. 4.7 and Fig. 4.8. At pH 2, six of 18 isolates tolerated and survived in 0.15% and 0.3% bile salts concentration. The result indicated that increasing bile salts concentration to 0.3% had a direct affect to the number of bacteria. All the same, pH too. The number of bacteria presents between 0.002 - 602 $\times 10^6$ CFU/ml. Most of the isolates were a high reduction of bacterial number after 2.5 h of the stress factor. Moreover, there was a different isolate namely isolate F31 that can tolerate to 4.0 h and had a bacterial number more than 3.0 log CFU/ml (Figure 4.7 and Figure 4.8).

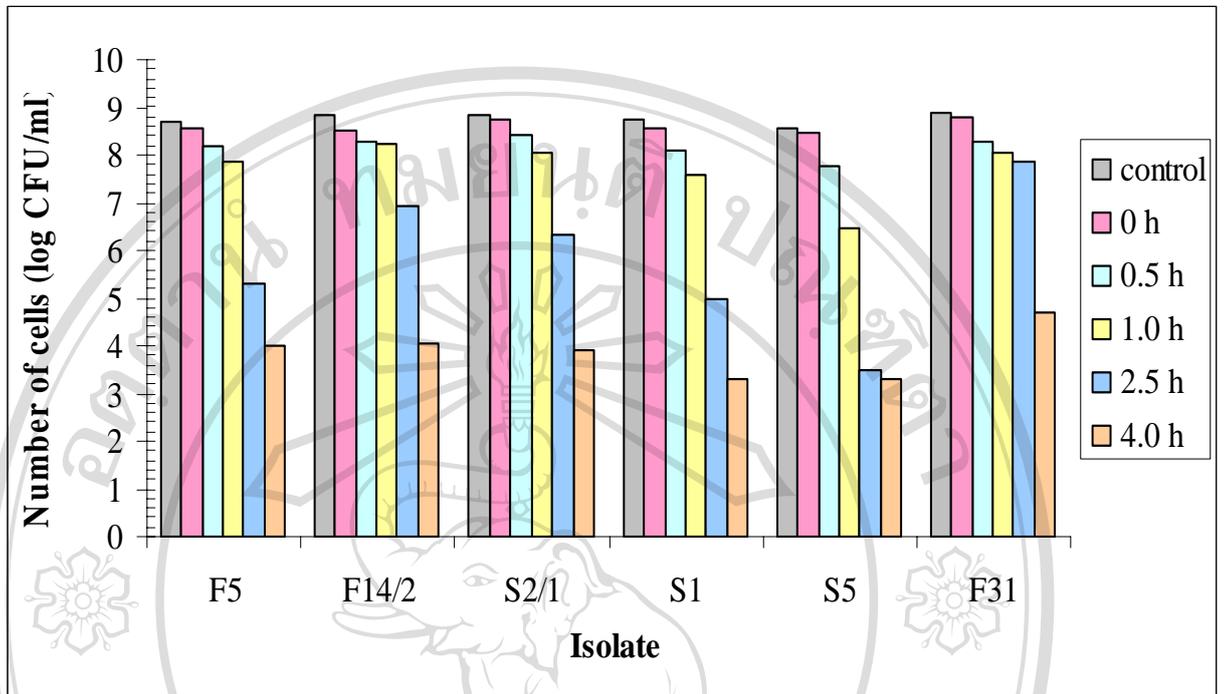


Figure 4.7 Number of lactic acid bacteria at pH 2, after incubation in MRS broth with 0.15% bile salts for 0, 0.5, 1.0, 2.5 and 4.0 h compare with control, MRS broth without bile salts.

Decrement of bacterial number happened after 0.5 h of incubation time in 0.15% bile salts. The number of viable bacteria at 0.15% bile salts were between 3.3 – 8.8 log CFU/ml. After 2.5 h the number of bacteria present between 3.48 – 7.86 log CFU/ml. Then, they decreased to at least 3.30 log CFU/ml (isolates S1 and S5).

But only isolate F31 remained 4.71 log CFU/ml that was the lowest reduction of viable cells at this condition.

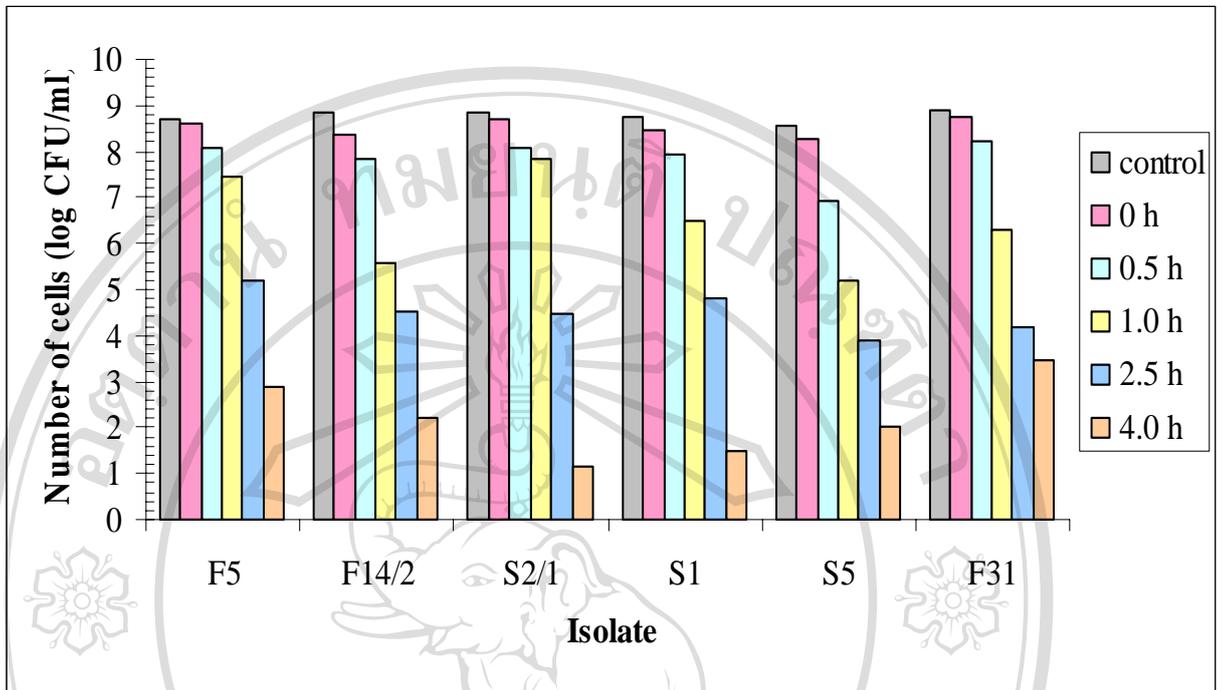


Figure 4.8 Number of lactic acid bacteria at pH 2, after incubation in MRS broth with 0.30% bile salts for 0, 0.5, 1.0, 2.5 and 4.0 h compare with control, MRS broth without bile salts.

After 0.5 h of incubation time in 0.30% bile salts, decreasing of bacterial number was happened. The number of viable bacteria at 0.30% bile salts were between 3.0 – 8.8 log CFU/ml. The number of bacteria were between 3.9 – 5.2 log CFU/ml after 2.5 h. Comparison with 0.15% bile salts, the number of viable bacteria were less than 0.15% bile salts. Moreover, most of isolates at 4.0 h had a viable bacteria less than 3.0 log CFU/ml. Exceptional, isolate F31 had more than 3.0 log CFU/ml.

It is important that probiotics microorganisms are able to reach the gastrointestinal tract (GIT) and remain viable there for 4 h. or more (Ouweland *et al.*, 1999). The result showed that it maintained an acceptable final cell concentration level as the time required for feed to pass through the digestive system is as short as

2.5 h. Probiotic bacteria vary considerably in their level of bile tolerance. Also, they explained the mechanism of tolerance is not understood and the minimum acceptable level of bile tolerance for a candidate probiotic remains unknown (Klaenhammer, 1982). Resistant to acid and bile salts are great importance in survival and growth of bacteria in the intestinal tract. So, it is a prerequisite for probiotics. Hence, we selected isolate F5, F14/2, S2/1, S1, S5 and F31 to identify in the genus level because they can tolerate at pH 2 in bile salts and have a number of bacteria between 4.0 -7.9 log CFU/ml as a critical concentration to screen for resistant strains.

4.4 Identification of acid and bile tolerant LAB strains

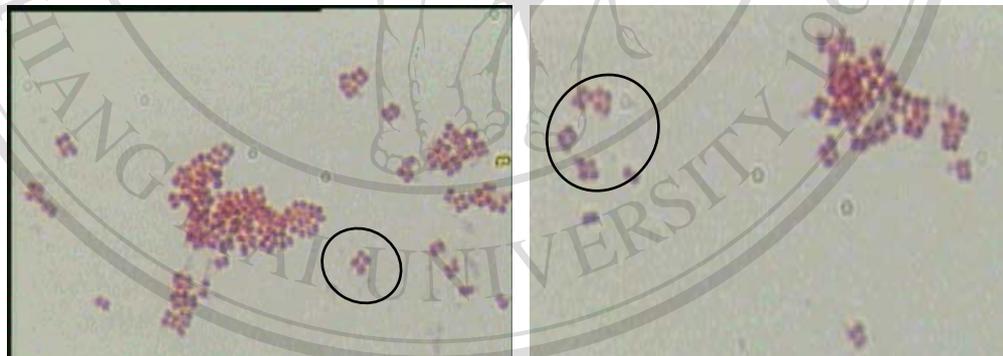
4.4.1 Morphological, phenotypic and biochemical methods

We selected 6 isolates (F5, F14/2, S2/1, S1, S5 and F31) of acid and bile tolerant lactic acid bacteria for identification to the genus level based on biochemical characteristics (Axelsson, 1993). The phenotypic characterization from biochemical test of 6 isolates of acid and bile tolerant lactic acid bacteria showed in table 4.8. All 6 isolates were Gram-positive and catalase negative. They were not produced CO₂ from glucose and not grew at 18% NaCl. They grew at 45°C, at 6.5% NaCl and at pH 4.4. At 10°C and pH 9.6, there were 2 isolates (isolate F14/2 and S1) that grew at this condition. Only isolates F14/2 and S1 showed tetrad arrangement.

Table 4.8 The phenotype of 6 isolates of acid and bile tolerant lactic acid bacteria

Isolate	F5	F14/2	S1	S2/1	S5	F31
Gram's staining	+	+	+	+	+	+
Tetrad form	-	+	+	-	-	-
Catalase test	-	-	-	-	-	-
CO₂ production	-	-	-	-	-	-
Growth						
- 45°C	+	+	+	+	+	+
- 10°C	-	-	-	+	+	-
- 6.5% NaCl	+	+	+	+	+	+
- 18% NaCl	-	-	-	-	-	-
- pH 4.4	+	+	+	+	+	+
- pH 9.6	-	+	+	-	-	-

Biochemical test indicated that all 6 isolates were cocci physiological characteristics. Isolate F14/2 and S1 had a tetrad form of arrangement, a specific form of the genus *Pediococcus* and *Aerococcus* (Figure 4.9). Normally, *Aerococcus* sp. and *Pediococcus* sp. are Gram-positive coccus, tetrad cluster arrangements. The bacteria do not form gas from glucose and all strains grow in broth containing 6.5% NaCl but do not grow at 10°C. These strains are non motile and catalase negative. The data in table 4.7 indicated that isolates F14/2 and S1 should be *Aerococcus* sp. or *Pediococcus* sp. Similarly, Phikunthong *et al.*, 2006 revealed that they isolated 80 lactic acid bacteria from *plaa-som*, a Thai fermented fish product mainly produced in central and north eastern regions of Thailand. Two isolates were *Aerococcus* spp. Moreover, isolates were determined as belonging to genera: *Lactobacillus* spp., *Pediococcus* spp., *Carnobacterium* spp. and *Enterococcus* spp. with the number of isolates 64, 14, 1 and 1, respectively.



F14/2

S1

Figure 4.9 Tetrad arrangement of isolate F14/2, on the left and isolate S1, on the right under light microscope at oil immersion.

Isolate S2/1 and S5 were Gram-positive, catalase-negative, not produced gas, by their ability to grow at 10°C and 45°C, in presence of 6.5% NaCl and at pH 4.4. So, they were identified as *Enterococcus* sp., most strains grow at 9.6 but isolate S2/1 and S5 not grow. The pH of the medium sustained and influenced on their survival ability. *Enterococcus* sp. can normally withstand pH ranges of between 4.0 and 9.6, depending on species (Mirtha, 2005). Perez *et al.*, 1982 reported that *E. durans* and *E. faecium* had maximum resistance at pH 6.6, while *E. faecalis* maximum survival was at pH of 6.6. In conclusion, there were 2 isolates of *Enterococcus* sp. from 16 isolates of acid and bile tolerant lactic acid bacteria that isolated from fecal samples of 2-4 months old healthy infant. Likewise, Khalil *et al.*, 2007 that identification and characterization of *Enterococcus faecalis*, *En. Faecium* and *En. durans* from 55 isolates of lactic acid bacteria from 3-6 old months infant fecal samples. Papamanoli *et al.*, 2003 revealed that 4% of 147 lactic acid bacteria that isolated from 2 types of naturally fermented dry sausages were *Enterococcus* sp.

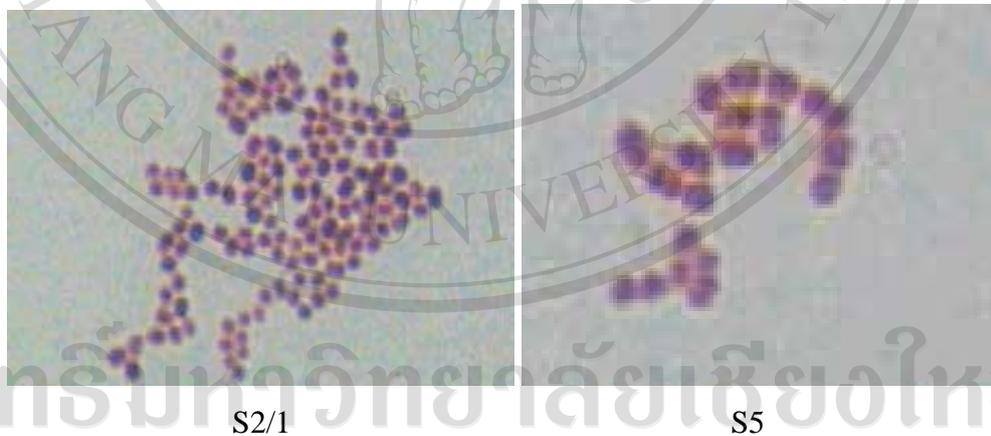


Figure 4.10 Cell arrangement of isolate S2/1, on the left and isolate S5, on the right under light microscope at oil immersion.

4.4.2 Identification at species level by 16s-rRNA Gene analysis

All 6 isolates were identified to species level. The 16S rRNA sequence coding region was amplified by PCR. The sequence of PCR products were determined directly with the prokaryotic 16S ribosomal DNA universal primer 27f and 1492r (Brosius *et al.*, 1978 and Weisburge *et al.*, 1991). The plasmid DNA that isolated from 6 isolates of acid and bile tolerant LAB was performed by agarose gel electrophoresis (Figure 4.11). The near full length nucleotide sequences of the 16S rRNA gene were determined. The nucleotide size of PCR products is about 1,500 bases on average. These partial nucleotide sequences of the PCR products were already registered in the DDBJ/GenBank/EMBL nucleotide databases under the following accession number; EU851939-EU851944. The sequences were compared with corresponding ones retrieved from the DDBJ/Genbank/EMBL nucleotide databases.

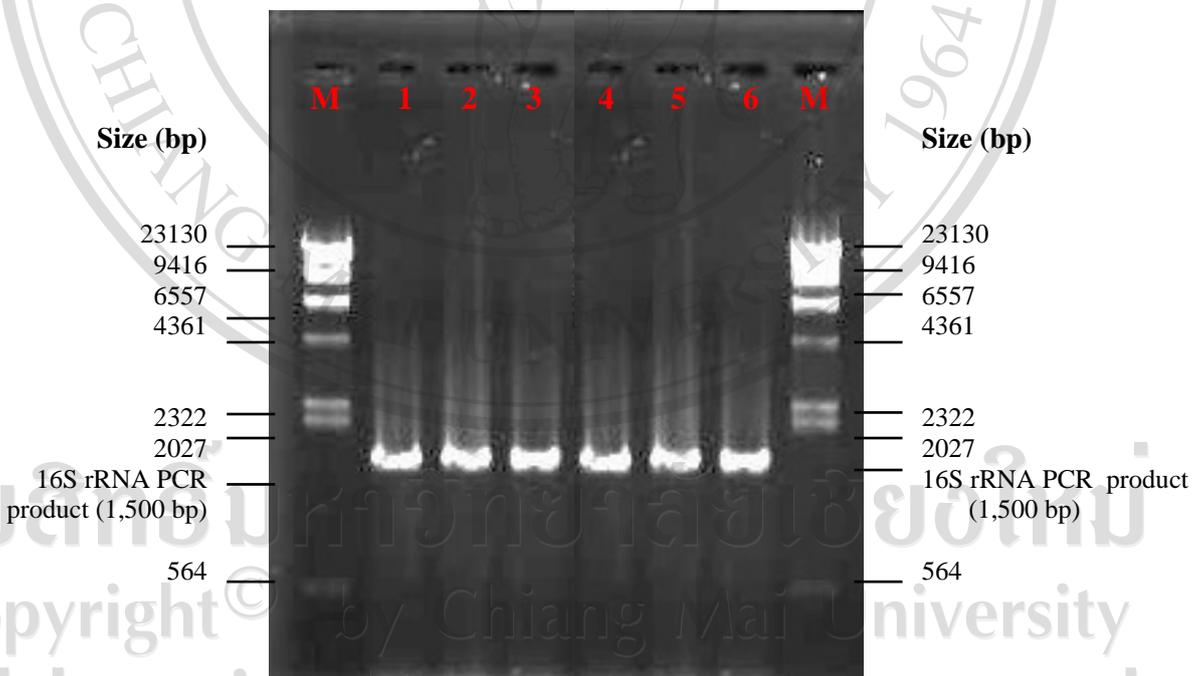


Figure 4.11 Agarose gel electrophoresis of PCR products from 6 isolates of acid and bile tolerant LAB. M, marker; Lane 1, isolate F14/2; Lane 2, isolate S2/1; Lane 3, isolate S1; Lane 4, isolate S5; Lane 5, isolate F5; Lane 6, isolate F31.

The phylogenetic tree was constructed by the rectangular cladogram method. The topologies of trees were evaluated by bootstrap analysis of the sequence data with CLUSTAL W software. More than 800 bases of 16S rRNA from isolate F14/2, S2/1, S1, S5, F5 and F31 were determined. The phylogenetic tree shown in Figure 4.12. Following phylogenetic analysis, representative isolate S1, S5, F5, F31 and F14/2 showed most closely *Pediococcus acidilactici* were the species most closely related to these 5 isolates. On the other hand, isolate S2/1 was closely related to genus *Enterococcus hirae*.

In this study, high sequence homologies were observed among *Pediococcus* species when identified these isolates from 16S rRNA sequences. *Pediococci* are often found living in nature, dairy products and foods produced by LAB (Cai *et al.*, 1999). Some isolates have been identified as *P. acidilactici* and *P. pentosaceus*. However, available phenotypic procedures to assign isolates to known species are difficult because it is not easy to differentiate clearly between species of *Pediococcus*. So, the representative isolate S1, S5, F5, F31 and F14/2 were identified as *Pediococcus acidilactici* as showed in the phylogenetic tree.

Other studies have shown the same result and found it difficult to identify strains to species level based on the sequencing results alone. Such as Devriese *et al* (1993) identified 10 strains of *Enterococcus* by their fermentative ability of carbohydrates using an API50CHL kit. As well as this study, biochemical results offered the information to distinguish the species with 16S rRNA sequences.

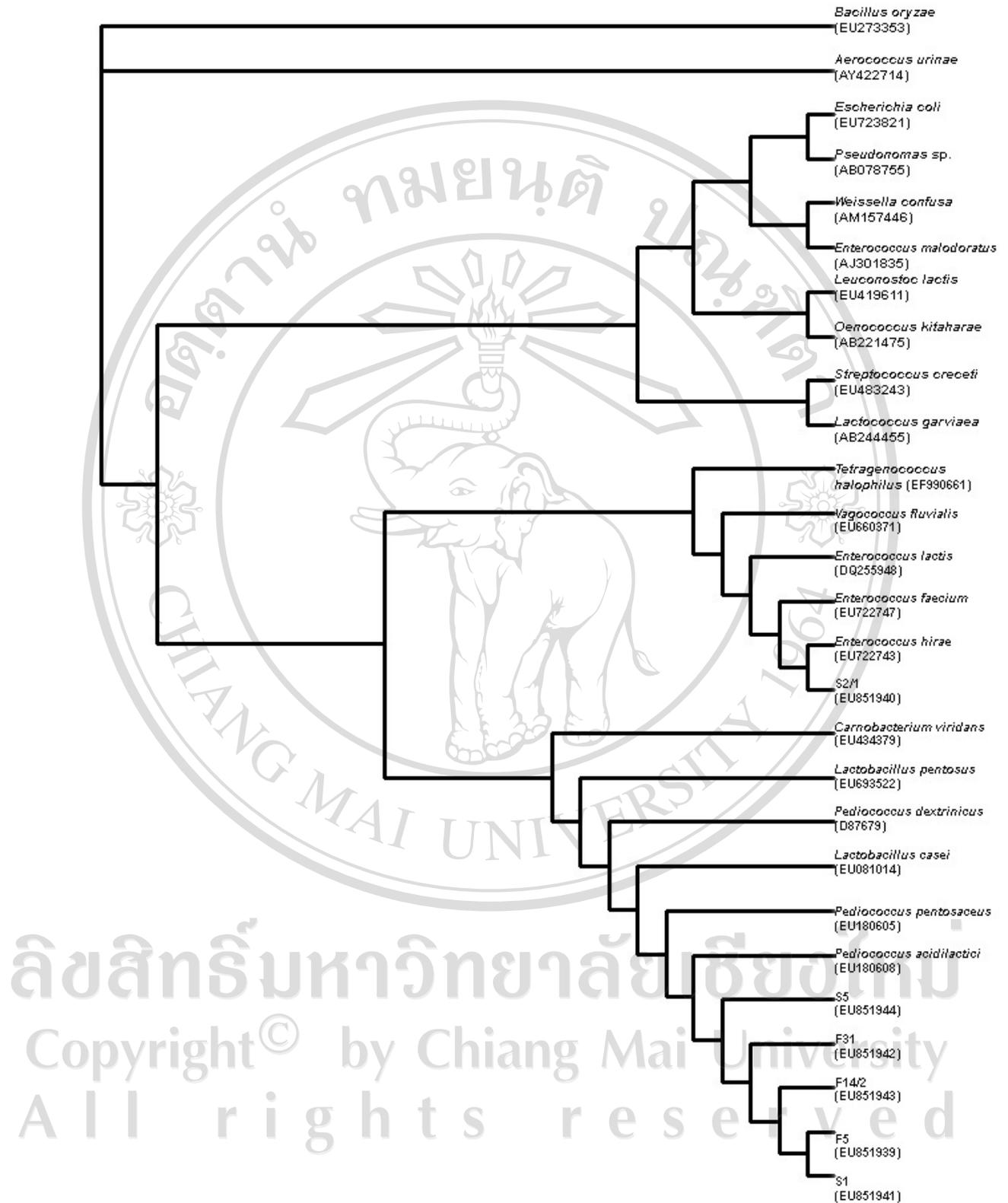


Figure 4.12 Phylogenetic tree show the position of 6 isolates acid and bile tolerant LAB. The tree was constructed by the rectangular cladogram method derived from the 16S rRNA gene sequences.