CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of lactic acid bacteria.

Lactic acid bacteria (LAB) play an essential role in food fermentation processes. The antimicrobial effect may be due to the production of organic acids (lactic and acetic acids), hydrogen peroxide and diacetyl, as well as bacteriocins or antimicrobial peptides (Daeschel, 1989).

Since bacteriocin-producing bacteria isolated from fermented vegetable and fruit products are well adapted to these conditions, they could ensure the safety and extend the shelf life of these foods. Therefore a search was made for antagonistic activities against food spoilage and pathogenic bacteria, in isolates from a range of fermented vegetable and fruit products.

Many studies have screened LAB isolated from fermented vegetable and fruit products; for example, in minimally processed fresh fruit and vegetables (William *et al*., 1998), Lactic acid fermented vegetable juices (Karovicova, 2004), Lactic acid fermentation of carrot, cabbage, beet, and onion vegetable mixtures (Nancy *et al*., 2000), Lactic acid bacteria isolated from olives fermented in Westhern Algeria for possible use probiotics (Kacem *et al*., 1998).

According to the results**,** isolation of lactic acid bacteria from ten kinds of fermented vegetable and fruit products (pickled green cabbage, bamboo shoot, soybean, wild spider flower, chinese radish, garlic, *Camellia olefera*, spanish plum, santol and mango) were collected from factories and local markets found that;

A total 126 isolates were LAB by change colour from bromocresol purple (violet) to yellow same report of Jutatip Boongird and Vichern Leelawatcharamas. They found 88 isolates were yellow colonies (Cause of change colour from other isolates produce acid). When test on MRS agar plus $CaCO_3$ compare with MRS agar plus microorganism test plates, found that 79 out of 126 isolates clear zone on MRS agar plus $CaCO_3$ bigger than clear zone on MRS agar plus microorganism, that mean effect from organic acid so we not require.

Twenty-six out of forty-seven isolates of LAB observed showed ability to inhibit the four indicator microorganisms *E. coli*, *B. cereus*, *S. aureus* and *S. enteritidis*. Only ten isolates were able to inhibit all four indicator microorganisms; thirteen isolates inhibited three indicator microorganisms and three isolates inhibited two indicator microorganisms. (Data shown in table 4.1, 4.2, 4.3), respectively.

Table 4.1 Inhibition of two indicator microorganisms by antimicrobial substance produced from lactic acid bacteria compare with clear zone on MRS agar plus Calcium carbonate ($CaCO_3$)

No.	Isolate	Clear zone size (m.m)				
		E. coli	***B. cereus***	***S. aureus***	***S. enteritidis***	**$CaCO_3$**
1	FH5-01/8	7	11	-	-	7.0
2	FH6-02/2	7.5	-	8.5	-	7.0
3	FH6-02/1	7	8	-	-	6.2

Table 4.1 showed three isolates on the inhibition of two indicator microorganisms by isolated FH5-01/8 and FH6-02/1 able inhibited *E.coli* TISTR73 and *B. cereus* were shown the clear zone size 7,11 and 7,8 millimeter respectively.

Moreover isolated FH6-02/2 could inhibited the growth of *E.coli* TISTR73 and *S. aureus* by shown clear zone size 7.5 and 8.5 millimeter respectively.

Table 4.2 Inhibition of three indicator microorganisms by antimicrobial substance produced from lactic acid bacteria compare with clear zone on MRS agar plus Calcium carbonate ($CaCO_3$)

No.	Isolate	Clear zone size (m.m)				
		E. coli	***B. cereus***	***S. aureus***	***S. enteritidis***	**$CaCO_3$**
1	FC4-01/3	7.6	11.0	7.6	-	7.0
2	FC2-01/4	10.0	8.3	9.0	-	7.0
3	FC6-01/1	8.3	8.3	-	7.5	7.0
4	FC2-01/2	8.6	6.6	-	6.65	6.4
5	FH5-01/4	8.1	8.25	7.0	-	6.2
6	FC5-01/4	8.3	7.0	6.5	-	7.0
7	FC6-01/3	8.0	8.0	10.0	-	7.0
8	FH5-01/2	8.0	7.16	7.3	-	6.0
9	FH6-02/3	7.5	7.3	7.3	-	5.0
10	FC5-01/2	7.3	6.3	7.3	-	6.3
11	FH5-01/6	7.25	6.25	-	6.0	6.0
12	FH5-01/5	6.6	6.1	7.3	-	6.1
13	FC4-01/7	6.5	-	7.0	6.0	5.5

Table 4.2 showed thirteen isolates on the inhibition of three indicator microorganisms by isolated FC4-01/3, FC2-01/4, FH5-01/4, FC5-01/4, FC6-01/3,

FH5-01/2, FH6-02/3, FC5-01/2 and FH5-01/5 could inhibited *E.coli* TISTR73, *B. cereus* and *S. aureus* were shown the clear zone. Moreover isolated FC6-01/1, FC2-01/2 and FH5-01/6 could inhibited the growth of *E.coli* TISTR73, *B. cereus* and *S. enteritidis*. Isolated FC4-01/7 disappear clear zone in *B. cereus* but also inhibited *E.coli* TISTR73, *S. aureus* and *S. enteritidis*.

Table 4.3 Inhibition of four indicator microorganisms by antimicrobial substance produced from lactic acid bacteria compare with clear zone on MRS agar plus Calcium carbonate ($CaCO_3$)

No.	Isolates	Clear zone size (m.m.)				
		E. coli	***B. cereus***	***S. aureus***	***S. enteritidis***	**$CaCO_3$**
1	FC4-01/4	9.3	7.75	7.16	13.25	6.0
2	FH6-01/2	9.8	7.6	9.5	7.6	6.5
3	FC6-01/2	9.6	9.4	8.8	8.5	7.0
4	FS2-01/6	8.3	9.3	8.0	8.5	8.0
5	FC6-01/4	8.5	7.5	8.16	6.5	6.0
6	FS2-01/3	7.25	7.25	7.0	8.0	7.0
7	FS5-01/4	7.6	7.0	7.3	7.5	6.0
8	FC4-01/1	7.0	7.3	8.3	7.5	6.5
9	FH6-02/4	7.3	6.75	6.6	6.75	5.5
10	FC5-01/3	7.8	7.74	7.16	7.5	5.5

Table 4.3 showed ten isolates were inhibited all of indicator microorganisms plate. The best isolates in this group FH6-01/2 inhibited *E.coli* TISTR73 and *S. aureus* by clear zone size 9.8 and 9.5 millimeter respectively. Furthermore isolate FC4-01/4 inhibited *B.cereus* and *S.enteritidis* were clear zone size 7.75 and 13.25 millimeter respectively.

All of Data from table 4.1 to 4.3 were pretest by paper disc method. The objective of the present work was to isolation and screening antimicrobial substance producing LAB. from fermented vegetable and fruit products. The result found that clearzone size on MRS agar plus $CaCO_3$ smaller than clearzone size in microorganism test plate, that mean not only acid but also other substance which able to inhibit indicator microorganism. Furthermore selection of extracellular antimicrobial substance producing LAB., found the clearzone from isolates non adjust pH bigger than the clearzone of isolates adjust pH to 7 (the clearzone disappear) because the antimicrobial substance that produced by this isolate work at the range of low pH.

Now we have many question with reference to antimicrobial substance producing LAB because Lactic acid bacteria are present in many fermented vegetable and inhibit other microorganisms through competition for nutrients and production of antimicrobial, such as lactic acid, hydrogen peroxide, and bacteriocins (Caplice and Fitzerald, 1999). These include many organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Doores, 1993). They have a very broad mode of action and inhibit both Gram-positive and Gram-negative bacteria as well as yeast and moulds (Blom and Mortvedt, 1991; Caplice and Fitzgerald, 1999). One good example is propionic acid produced by propionic acid bacteria, which has formed the basis for some biopreservative products, given its antimicrobial action against microorganisms including yeast and moulds. Microgard is a Food and Drug Administration (FDA)-approved fermentate produced by Propionibacterium

freudenreichii subsp. shermanii which contains propionic acid and is used in an estimated 30% of the cottage cheese manufactured in the United States (Daeschel, 1989). In addition to acids, starter strains can produce a range of other antimicrobial metabolites such as ethanol from the heterofermentative pathway, H_2O_2 produced during aerobic growth and diacetyl which is generated from excess pyruvate coming from citrate (Ray and Daeschel, 1992). In particular, H_2O_2 can have a strong oxidizing effect on membrane lipids and cellular proteins and is produced using such enzymes as the flavo protein oxidoreductases NADH peroxidase, NADH oxidase and a-glycerophosphate oxidase (Condon, 1987). Obviously, each antimicrobial compound produced during fermentation provides an additional hurdle for pathogens and spoilage bacteria to overcome before they can survive and/or proliferate in a food or beverage, from time of manufacture to time of consumption. Since any microorganism may produce a number of inhibitory substances, its antimicrobial potential is defined by the collective action of its metabolic products on undesirable bacteria.

Other examples of secondary metabolites produced by LAB which have antagonistic activity include the compound reuterin (Axelsson *et al*., 1989; Chung *et al*., 1989; Talarico and Dobrogosz, 1989) and the recently discovered antibiotic reuterocyclin (Ganzle et al., 2000; Holtzel *et al*., 2000), both of which are produced by strains of Lactobacillus reuteri. Reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of h-hydroxypropionaldehyde. It has a broad spectrum of activity and inhibits fungi, protozoa and a wide range of bacteria including both Gram-positive and Gram-negative microorganisms.

More recently, the first antibiotic produced by a LAB was discovered (Ganzle *et al*., 2000; Holtzel *et al*., 2000). Reuterocyclin is a negatively charged, highly hydrophobic antagonist, and structural elucidation revealed it to be a novel tetramic acid. The spectrum of inhibition of the antibiotic is confined to Grampositive bacteria including *Lactobacillus* spp., *Bacillus subtilis*, *B. cereus*, *E. faecalis*, *S. aureus* and *Listeria innocua*. Interestingly, inhibition of *E. coli* and *Salmonell*a *enteritidis* is observed under conditions that disrupt the outer membrane, including truncated LPS, low pH and high salt concentrations. (Stevens et al., 1992).

So in order to answers, we according apply the method and confirm test for the next step.

4.2 Screening of antimicrobial substance producing LAB

Now report, detection *Salmonella* spp. from fresh vegetable, salads, melons, sprouts, tomatoes, and other fruit. (Arvind, 2002). Fruits and vegetables are grown in every state, but California, Florida found 2006 *E. coli* contamination problems in pre-cut spinach shipped and many paper report found *B. cerus* and *S. aureus* in vegetable. So we choose 4 bacteria for indicator microorganismtest.

Forty-seven isolates of LAB that were isolated from 50 samples of fermented vegetable and fruit products were used for screening of antimicrobial substance producing lactic acid bacteria. Twenty-six of 47 isolates were examined as LAB produced potential inhibitory substance againt indicator microorganisms, *E.coli* TISTR73, *B. cereus*, *S.aureus* and *S. enteritidis*. These 26 isolates were selected as antimicrobial substance producing LAB because they showed the clear inhibition zones around the paper disc on test medium. The results show that 10 isolates were inhibited all of four indicator microorganisms as show in number 1 to 10 of table 4.3. while other 13 isolates and 3 isolates inhibited only 3 and 2 of indicator microorganisms as show in Table 4.2 and 4.1 respectively.

It was found that clear zone of these 26 isolates on MRS agar plus indicator microorganisms are bigger than the clear zone in MRS agar plus $CaCO_3$ that mean it should have other of antimicrobial substance produced by LAB.

4.3 Selection of antimicrobial substance producing LAB

After screening of antimicrobial, 26 isolate as positive isolates, the concentrated supernatant of those isolates checked by paper disc diffusion assay. To findout other antimicrobial substance in the supernatant of cultured broth, we could

designed separated concentrated supernatant into two part, non-adjusted pH and adjusted pH to 7 with 3 N NaOH, for eliminate the effect before apply to paper disc.

It was found that the non-adjust pH concentration 26 able to inhibit all indicator microorganism by showed the clear zone appear on MRS agar plate plus with each indicator microorganism *E.coli* TISTR73, *B. cereus*, *S.aureus* and *S. enteritidis*. But the concentrated adjust to pH 7.0 did not showed the clear zone on MRS plus indicator microorganisms. So we predicted that the antimicrobial substance that produce by thus 26 isolate may need low condition for work as the previous reported the bacteriocin was active over a wide range between 2 and 10 (Noonpakdee *et al*., 2003), According to Pucci *et al*.(1998) the result presented activity of a bacteriocin produce by *Pediococcus acidilactici* PAC 1.0 strains could growth at pH 5.5 to 7.0 in ATP broth. Thus we need other method to determined the type of antimicrobial substance of these 26 isolates.

4.4 Determination of antimicrobial substance

Over the past few years, the most studies of anti-bacterial LAB have test pure culters in well or disc diffusion assays (Jeppesen and Huss, 1993a). The results of depend on each method for detect antimicrobial. In this study, In this study, the results of the disc diffusion assays showed that 26 isolates produce antimicrobial substances. All 26 isolates were chosen for determination of antimicrobial substance study. Agar well diffusion techniques were used to determination the substance from those isolates. The results of well diffusion assay showed that the concentrated supernatant from isolates were depressed with 0.5 g/ml (w/v) β-glycerophosphate. While 7 isolates were not depressed with 0.5 g/ml (w/v) β-glycerophosphate and showed the clearzone around the well.

From these twenty-six isolates resulted in the loss of ability to inhibit the indicator microorganisms of nineteen isolates, indicating that the bactericidal activity was the result of lactic acid (Brasil, 2004).

Thus it was possible that the other seven isolates are bacteriocin-producing microorganisms.

4.5 Determination of bacteriocin producing LAB

Then proteinase K and β-glycerophosphate were added to the reaction. It was found that 3 isolates, FC4-01/1, FC4-01/4 and FC6-01/2, of 7 isolates lost their inhibitory ability against indicator microorganisms, because the bacteriocin which produced by these 3 LAB was degraded by proteinase K and the effect of lactic acid was omitted by β-glycerophosphate indicating the presence of bacteriocin (Table 4.4).

Isolates FC4-01/1 and FC4-01/4 were from pickled cabbage and could inhibit of all indicator microorganisms (Figure 4.1, 4.2 and 4.3). FC6-01/2 was able to inhibit two indicator microorganisms, *Staphylococcus aureus* and *Salmonella enteritidis*. All three isolates were identified as *Lactobacillus* sp.(Table 4.5).

Similar to Peeva et al. (2006) report the protein nature of the antimicrobial agent in test the producer strain cultures was demonstrated by treatment of 500 µl prepared culture supernatant with 25 µl proteinase K solution (20 mg/ml) for 2 houurs at 37^0C. It was found protein nature of the bacteriocin-like substance loss of inhibitory activity after treatment with proteinase K. There for of 7 isolates are bacteriocin-producing isolates originated from fermented vegetable and fruit products.

Table 4.4 Screening of bacteriocin from antimicrobial substance producing LAB

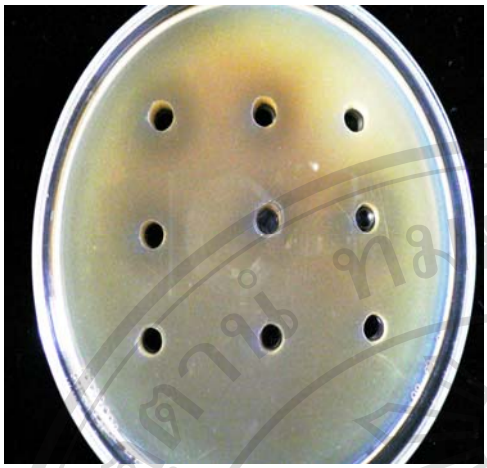
Isolates	Clear zone size on MRS agar plus Indicator strains (mm.)											
	E. coli			*B. cereus*			*S. aureus*			*S. enteritidis*		
	S+ H_2O	S+ β-gly	S+ β-gly + ProK	S+ H_2O	S+ β -gly	S+ β-gly + ProK	S+ H_2O	S+ β -gly	S+ β -gly + ProK	S+ H_2O	S+ β -gly	S+ β -gly+ ProK
FH6-01/2	+	-	-	+	-	-	+	-	-	+	-	-
FC4-01/1	+	+	-	+	+	-	+	+	-	+	+	-
FH6-02/4	+	-	-	+	-	-	+	-	-	+	-	-
FC4-01/4	+	+	-	+	+	-	+	+	-	+	+	-
FC6-01/2	+	-	-	+	-	-	+	+	-	+	+	-
FS2-01/6	+	-	-	+	-	-	+	-	-	+	+	-
FS2-01/3	+	-	-	+	-	-	+	-	-	+	-	-

+, positive; -, negative

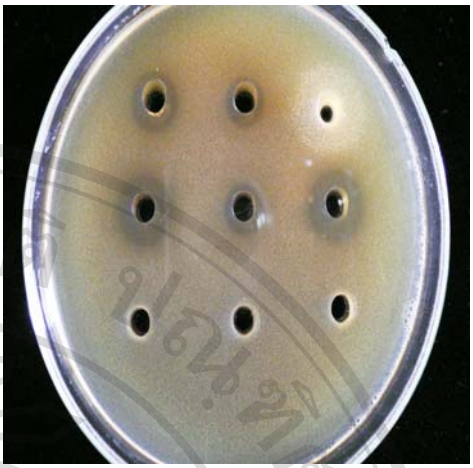
The data in table 4.4 shown the effect from β-glycerophosphate to decresed effect from organic acid. All of isolates, sample plus H_2O (the clearzone appear). Moreover sample plus Beta-glycerophosphate (three isolates the clearzone appear) that mean not effect from organic acid but the effect from bacteriocin.

Kim and Worobo (2000) used 2% β-glycerophosphate (Sigma Chemical Co.) for the bacterial counts were determined with MRS plates. The report was found *Lactobacillus acidophilus* to be the least acid tolerant.

Similarly Renata Bromberg (2004) test rule out any inhibition due to pH reduction cause by organic acid production by used 2% sodium Beta-glycerophosphate was added to MRS agar.

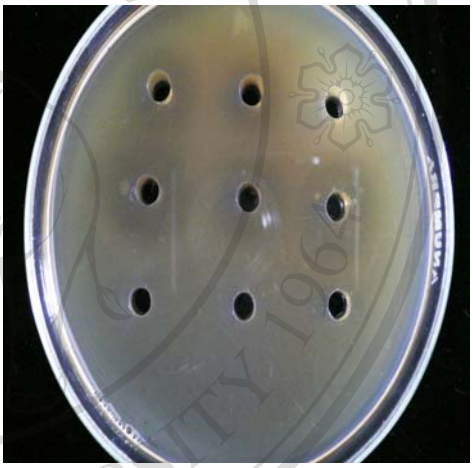


E. coli



B. cereus

S. aureus



S. enteritidis

1	2	3
4	5	6
7	8	9

Figure 4.1 Bacteriocin property of FC4 - 01/1 on the inhibition of 4 indicator microorganisms.

1 = sample + H_2O 2 = sample+ H_2O + β-gly. 3 = sample + β-gly. + Pro K

4 = sample+H_2O (boiled) 5 = sample + H_2O + β-gly. (boiled) 6 = sample+H_2O+Pro K

7 = H_2O + β-gly 8 = H_2O + pro K 9 = H_2O + β-gly.+Pro K

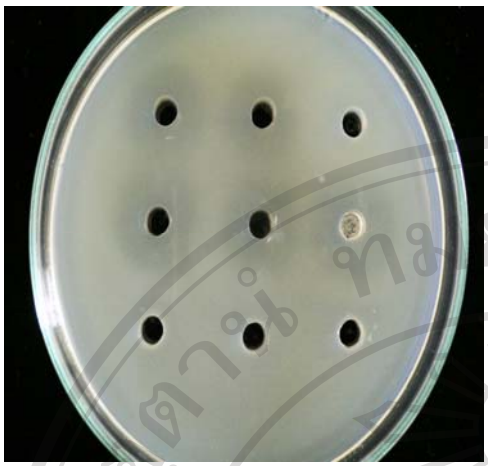
The term bacteriocin was introduced by Jacob and coworker (1953), it was defined as protein antibiotics of relative high molecular weight mainly working against the same, or closely related species by adsorption to receptors on the target cells.

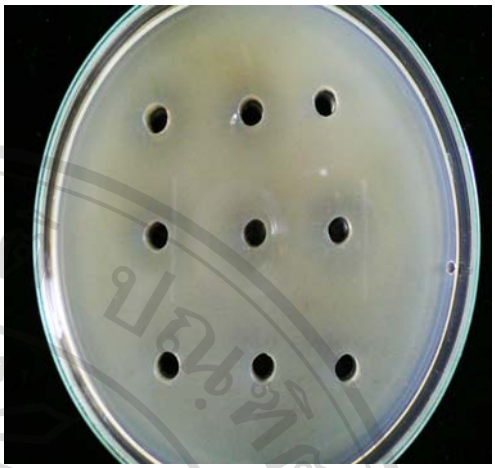
From the pretest by paper disc method, it could not summarize the antimicrobial substance producing LAB were effect from bacteriocin so, The confirm test by Agar well diffusion assay applied by added β-glycerophosphate for decress effect from organic acid.

The result found that seven isolates out of twenty-six isolates were may bacteriocin-producing microorganisms.

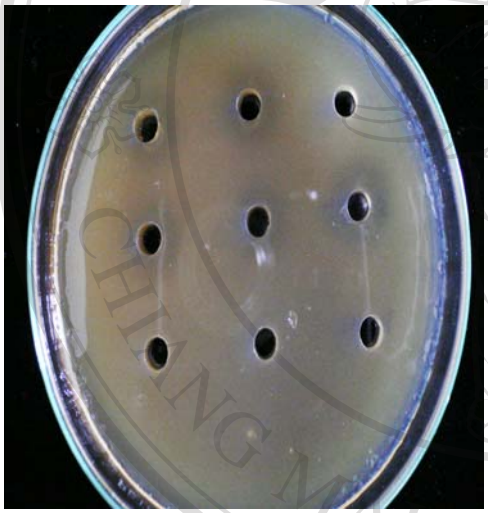
Morover bacteriocin were proteins so, Purified bacteriocin was assessed for its sensitivity to various enzymes. Faruk (2002) test sensitivity to proteolytic enzyme by treated with proteinase K, pronase E and Trypsin. Similarly Kelly (1996) test sensitivity to heat and proteolytic enzymes by application Trypsin T-4799 and Proteinase K at final concentration of 1 mg/ml. led to inactivation of the antagonistic activity of culture.

When test sensitivity to proteolytic enzyme by treated with proteinase K (final concentration of 1 mg/ml) found three isolates were bacteriocin. An example in figer 4.1 shown the ability of bacteriocin FC4-01/1 on the inhibition of 4 indicator microorganism. The third row 7, 8, and 9 agar well were control disappear clearzone but the first and the fourth agar well sample plus distillwater shown the clearzone that mean this isolate was heat-resistant. Furthermore the second and the fifth agar well, sample plus distillwater plus β-glycerophosphate shown the clearzone. Morever the third agar well, sample plus Beta-glycerophosphate and plus proteinase K (final concentration of 1 mg/ml) disappear clearzone so we can concould this isolates was bacteriocin. Also isolates FC4-01/4 and FC6-01/2 were bacteriocin.





E. coli ***B. cereus***



S. aureus ***S. enteritidis***

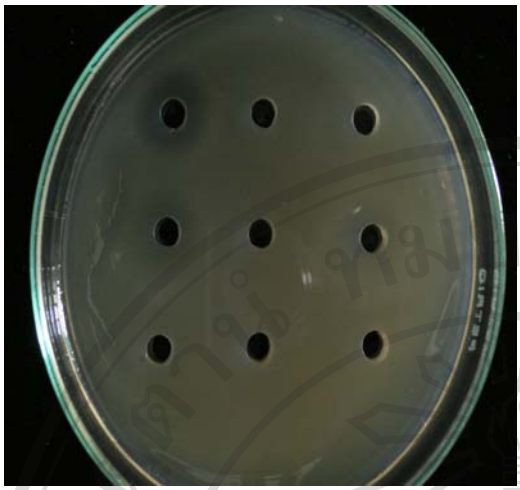
1	2	3
4	5	6
7	8	9

Figure 4.2 Bacteriocin property of FC4 - 01/4 on the inhibition of 4 indicator microorganisms.

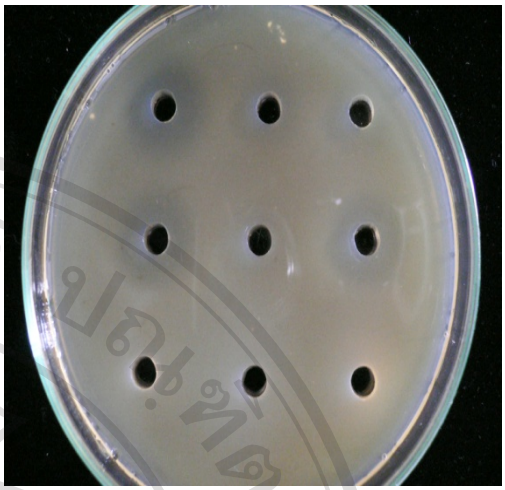
1 = sample + H_2O 2 = sample+ H_2O + β-gly. 3 = sample + β-gly. + Pro K

4 = sample+H_2O (boiled) 5 = sample + H_2O + β-gly. (boiled) 6 = sample+H_2O+Pro K

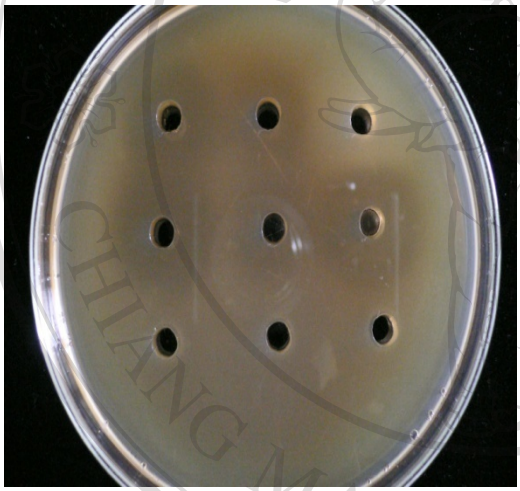
7 = H_2O + β-gly 8 = H_2O + pro K 9 = H_2O + β-gly.+Pro K



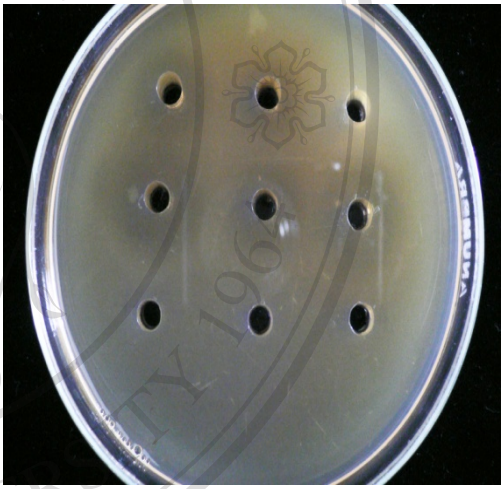
E. coli



B. cereus



S. aureus



S. enteritidis

1	2	3
4	5	6
7	8	9

Figure 4.3 Bacteriocin property of FC6 - 01/2 on the inhibition of 2 indicator microorganisms.

1 = sample + H_2O 2 = sample+ H_2O + β-gly. 3 = sample + β-gly. + Pro K

4 = sample+H_2O (boiled) 5 = sample + H_2O + β-gly. (boiled) 6 = sample+H_2O+Pro K

7 = H_2O + β-gly 8 = H_2O + pro K 9 = H_2O + β-gly.+Pro K

Figure 4.2 shown bacteriocin property of FC4 - 01/4 on the inhibition of 4 indicator microorganisms.

The clear zone of isolate FC4-01/4 detect on each plate show the antimicrobial ability on the inhibition of 4 indicator microorganisms. The clear zone not appear when test with β-glycerophosphate and proteinase K (3). While it was found that clear zone still detected on Agar well 2, 5 and 6. That mean isolate FC4-01/4 capable to produce both of bacteriocin and other antimicrobial because after added proteinase K clear zone still appear on plate. Therefore, clear zone detected on plate as result of other antimicrobial not action of bacteriocin. However, isolate FC4-01/4 still activity when test with β-glycerophosphate that mean clear zone appear it not effect of organic acid. Not only isolated FC4-01/4 produce antimicrobial like-bacteriocin but also found that some of antimicrobial were able to indicator microorganisms.

In case of isolate FC6-01/2 (Figure 4.3) on the inhibited of 2 indicator microorganisms. Similar result were obtain by describe above. But this isolate was not produce bacteriocin active against *E. coli* TISTR73 and *B. cereus*. Because clear zone detect on plate of *S. aureus* and *S. enteritidis*.

4.6 Acid Tolerance

The three isolates, bacteriocin producing LAB that have fermented vegetable and fruit products, were test for acid and bile toterance. The acid and bile tolerance of LAB is dependent upon the pH profile and the composition of cytoplasmic membrane, which is largely influenced by the type of bacteria, type of growth media, and the incubation condition (Havenaar *et al*., 1992; Hood and Zottola, 1998). Acid tolerance is a fundamental property that indicates the ability of probiotic microorganism to survive passage through the stomach (Prasad *et al*., 1998; Park *et al*, 2002).

All experiments Acid Tolerance test found that all of three isolates (FC4-01/1, FC4-01/4 and FC6-01/2) could best growth at pH 4, pH3 and pH2 respectively, when compare with control. (data shown in table 4.5). Generally, survival was low at pH 0.5, 1 and 2 moderate at pH 3 and good at pH 4 and 5 (Jin *et al*., 1998). However, all of three isolates had a moderate survival rate at pH 3 and good survival rate at pH 4. Survival of these isolates is presented in table 4.5. The all of isolates proved to be most acid tolerance. Little was seen at pH 2 with these isolate. The similar to the result obtain by Goldin, Gorbach (1992) with *Lactobacillus* GG which is regarded as a probiotic.

4.7 Bile tolerance

Bile tolerance is one of the most essential criteria for a strain to be a probiotic culture (Gilland, 1979). Bile salts are surface active chemicals produce in the liver from the catabolism of chlolesterol (Brandt and Bernstein, 1976). In this study found that all of three isolates FC4-01/1, FC4-01/4 and FC6-01/2 could growth at concentration 0.15% of bile salt better than 0.3% when compare with control (Data shown in table 4.6)

Table 4.5 Acid Tolerance

Isolates	Colony (CFU/ml)				Log CFU/ml			
	control	pH2	pH3	pH4	control	pH2	pH3	pH4
FC4-01/1	$2.50x10^{13}$	$7.6x10^{12}$	$1.12x10^{13}$	$2.12x10^{13}$	13.39	12.88	13.05	13.32
FC4-01/4	$2.36x10^{13}$	$5.3x10^{12}$	$1.32x10^{13}$	$1.64x10^{13}$	13.37	12.72	13.12	13.21
FC6-01/2	$2.02x10^{12}$	$6.5x10^{11}$	$1.25x10^{12}$	$1.75x10^{12}$	12.30	11.81	12.09	12.24

Table 4.6 Bile Tolerance

Isolates	Time (h)	Colony (CFUml)			Log CFU/ml		
		Control	0.15% bs	0.3% bs	Control	0.15% bs	0.3% bs
FC4-01/1	0	$2.09x10^8$	$2.02 x10^8$	$1.98 x10^8$	8.32	8.30	8.29
	0.5		$1.93 x10^8$	$1.25 x10^8$		8.28	8.09
	1		$1.46 x10^8$	$1.12 x10^8$		8.16	8.04
	2.5		$1.67 x10^8$	$9.6x10^7$		8.22	7.98
	4		$1.81 x10^8$	$8.0 x10^7$		8.25	7.90
FC4-01/4	0	$2.07x10^8$	$1.98x10^8$	$1.62 x10^8$	8.31	8.29	8.21
	0.5		$1.65 x10^8$	$1.39 x10^8$		8.21	8.14
	1		$1.73 x10^8$	$9.9 x10^7$		8.23	7.99
	2.5		$2.16 x10^8$	$1.17 x10^8$		8.33	8.06
	4		$2.24 x10^8$	$1.53 x10^8$		8.35	8.18
FC6-01/2	0	$2.08x10^8$	$1.97 x10^8$	$1.54 x10^8$	8.31	8.29	8.18
	0.5		$1.89 x10^8$	$1.61x10^8$		8.27	8.20
	1		$1.32 x10^8$	$7.3x10^7$		8.12	7.86
	2.5		$1.92 x10^8$	$9.1 x10^7$		8.28	7.95
	4		$2.06 x10^8$	$1.34 x10^8$		8.31	8.12

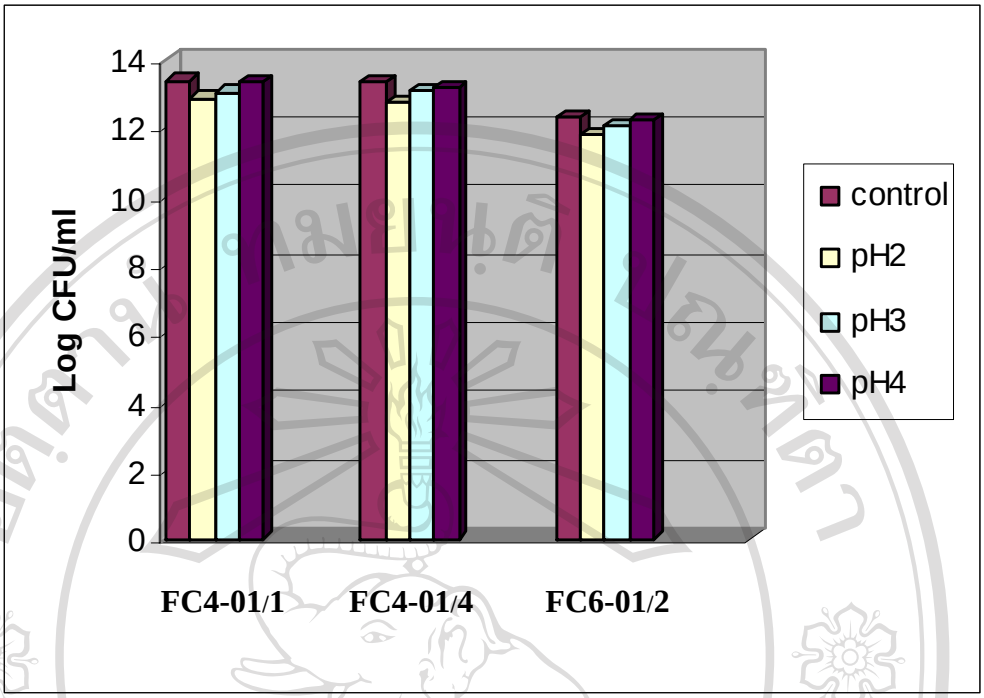


Figure 4.4 Acid tolerance of FC4-01/1, FC4-01/4 and FC6-01/2

Isolates FC4-01/1, FC4-01/4 and FC6-01/2 were found to be the best acid tolerant when compare with control. Isolate FC4-01/1 with a final population of 12.88, 13.05, 13.22 log cfu/ml in pH 2, 3 and 4 respectively, The population of isolate FC4-01/4 show final population of 12.72, 13.12, 13.21 log cfu/ml in pH 2, 3 and 4 respectively, whereas isolates FC6-01/2 was found to be the least acid tolerance at pH 2, 3 and 4 with a final population of 11.81, 12.09, 12.24 log cfu/ml respectively.

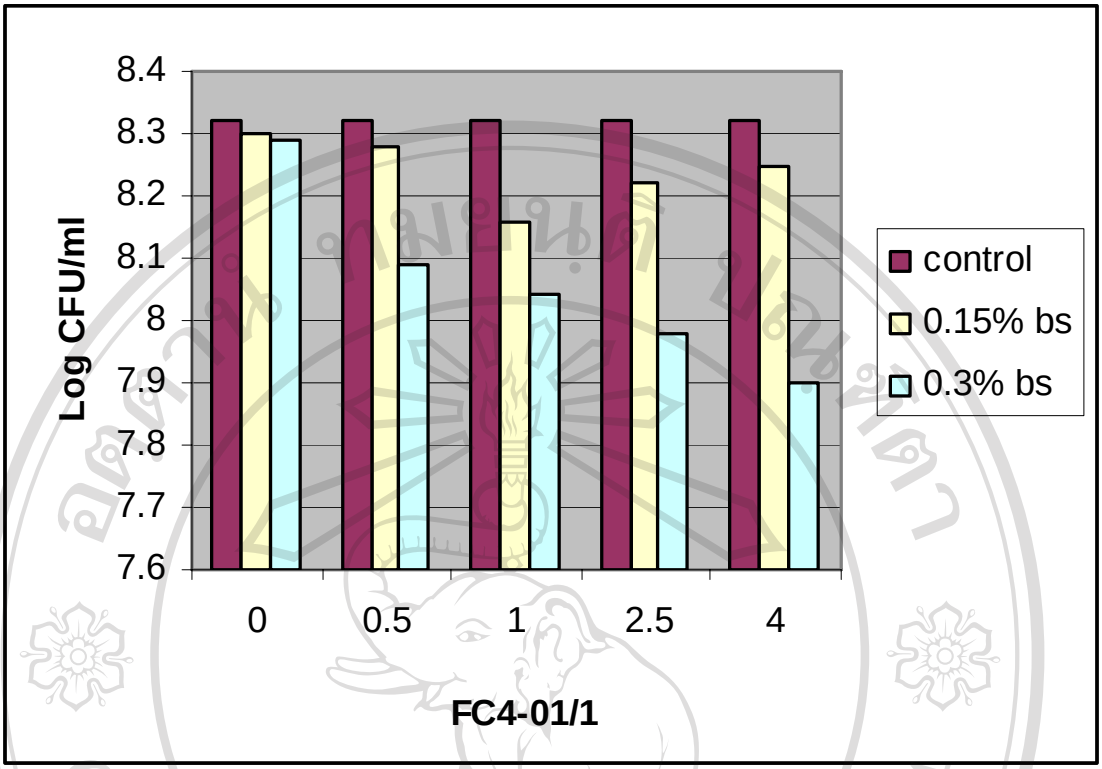


Figure 4.5 Bile Tolerance of FC4-01/1

All experiments were replicated five time, and each viable cell count performed in figure 4.5. Isolate FC4-01/1, a relatively high population of 0.15% bile salt, with a final popular 8.30 log cfu/ml. Bile salt 0.3% was found to be the least bile tolerance, with a final population of 7.90 log cfu/ml. These isolate, with 0.15 % bile salt was growth at 0 h. and drop to 1 h. After could growth agints same the *L. acidophilus* NCFM (Kim, 2000). While these isolate, with 0.3 % bile salt best growth at 0 and droop to 4 h. same the *L. acidophilus* 30SC (Kim, 2000).

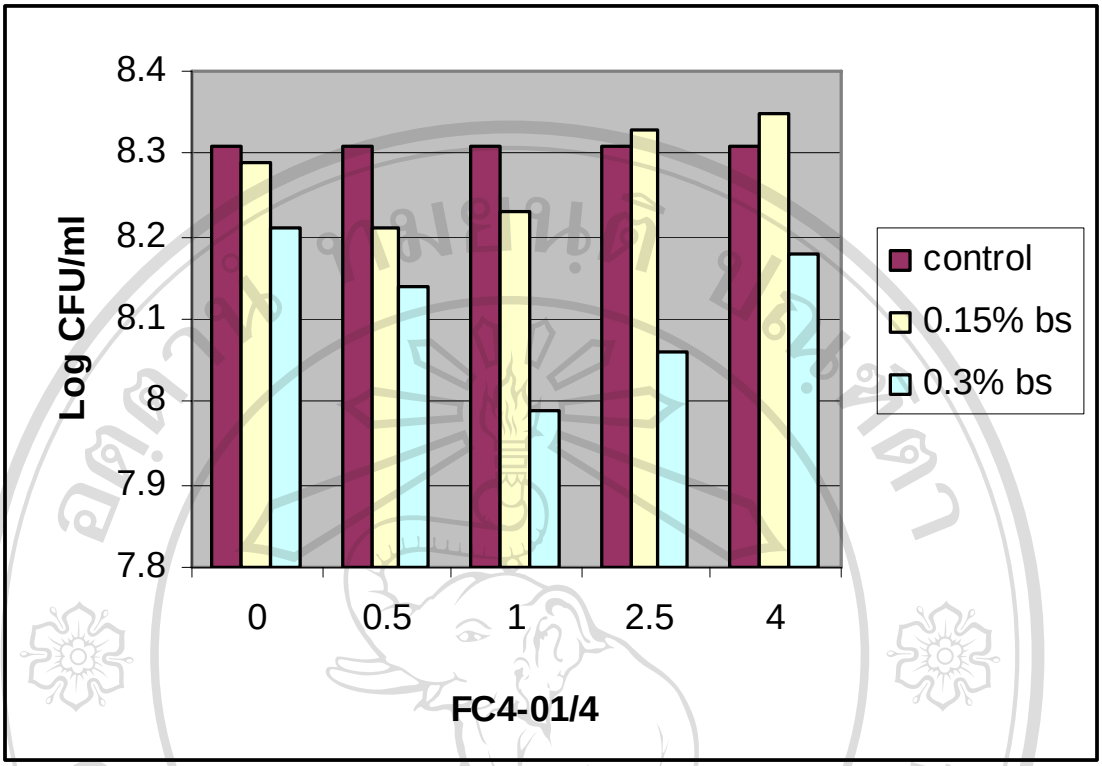


Figure 4.6 Bile Tolerance of FC4-01/4

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Isolate FC4-01/4, a relatively high population of 0.15% bile salt, with a final popular 8.35 log cfu/ml. Bile salt 0.3% was found to be the least bile tolerance, with a final population of 7.99 log cfu/ml.

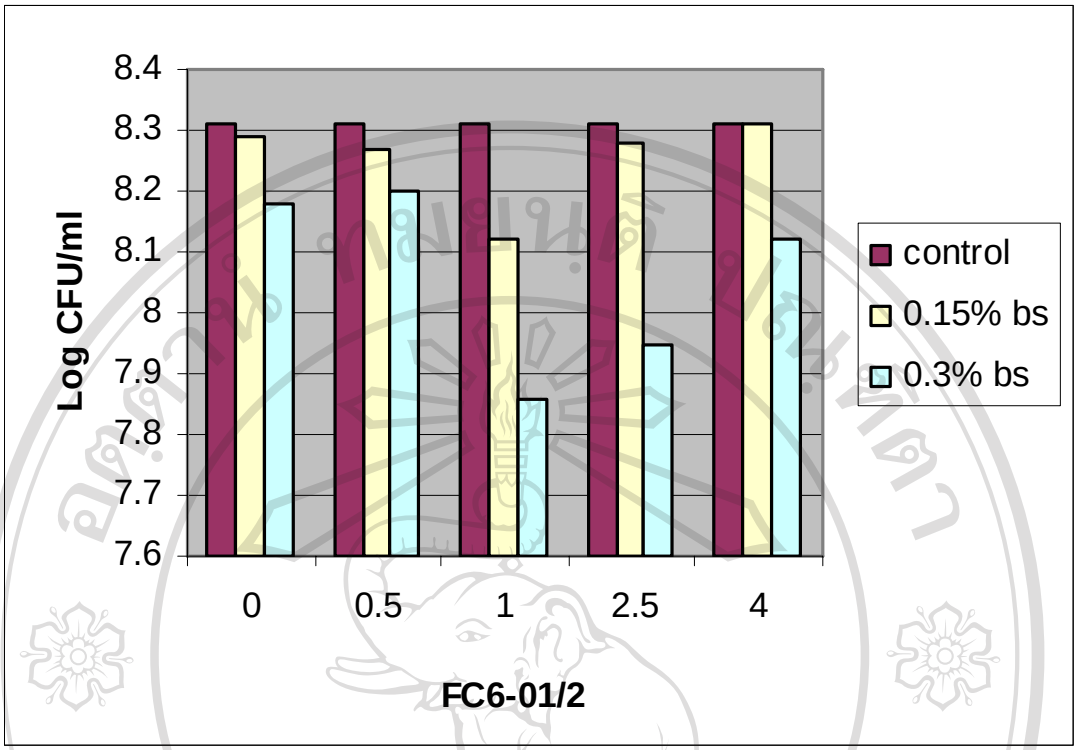


Figure 4.7 Bile Tolerance of FC6-01/2

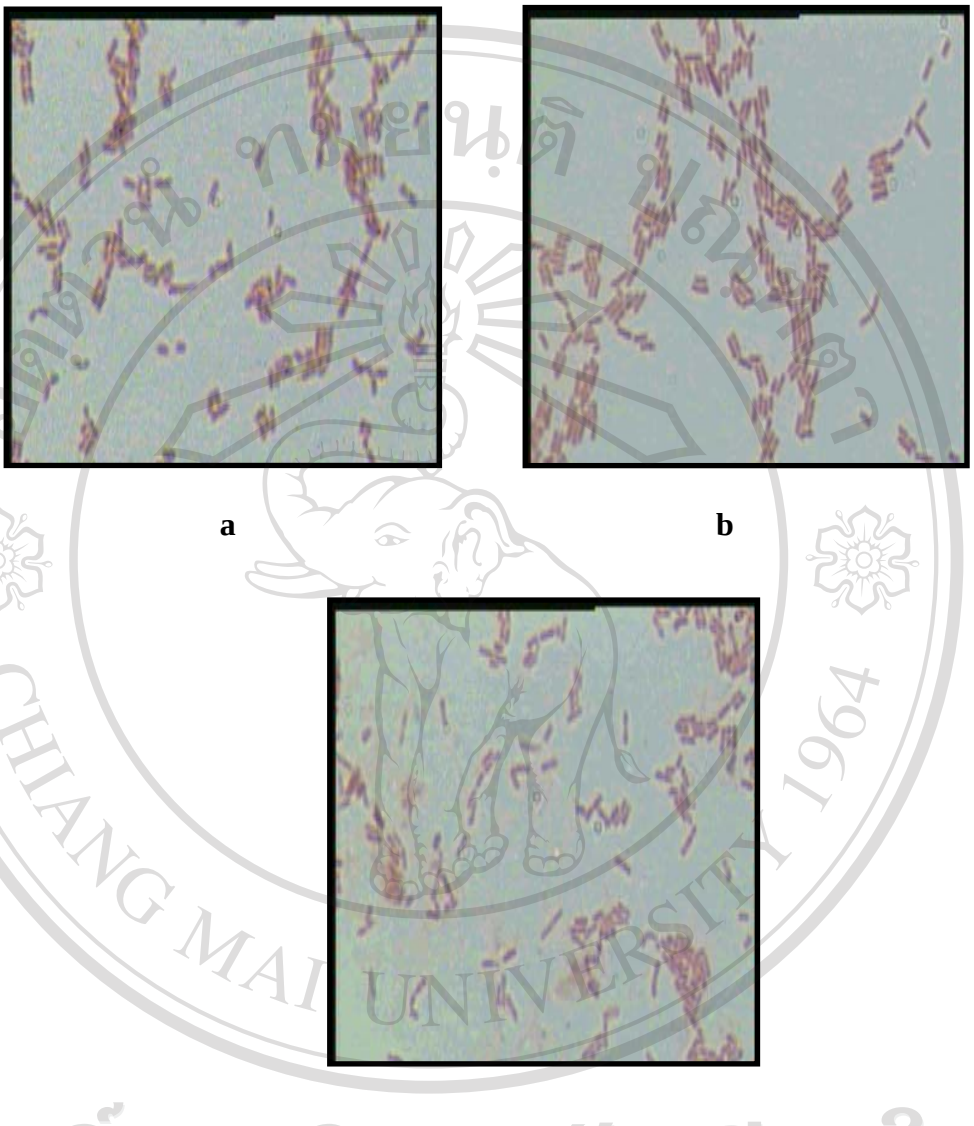
Isolate FC6-01/2, a relatively high population of 0.15% bile salt, with a final popular 8.29 log cfu/ml. Bile salt 0.3% was found to be the least bile tolerance, with a final population of 7.86 log cfu/ml.

4.8 Physiologycal characterization of bacteriocin-producing LAB

We selected 3 isolates (FC4-01/1, FC4-01/4 and FC6-01/2) that are acid and bile lactic acid bacteria were identified to the genus level based on the following key biochemical characteristics (Axelsson, 1993). The phenotypic characterization from biochemical test of 3 isolates of acid and bile tolerant lactic acid bacteria showed in table 4.5 and 4.6. All 3 isolates were Gram-positive and catalase negative. They not produce CO_2 from glucose and not growth 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 3 isolates that showed rods form arrangement.

Table 4. 7 Biochemical characters of three isolates from fermented vegetable and fruit products.

Character / **Isolates**	**Rods**		
	FC4-01/1	**FC4-01/4**	**FC6-01/2**
Cell	rod	rod	rod
Tetrad formation	-	-	-
CO_2 from glucose	-	-	-
Growth at 10^0C	+	+	+
Growth at 45^0C	+	+	+
Growth at 6.5% NaCl	+	+	+
Growth at 18% NaCl	-	-	-
Growth at pH 4.4	+	+	+
Growth at pH 9.6	-	-	-
Lactic acid	ND	ND	ND
Genus	*Lactobacillus* sp.	*Lactobacillus* sp.	*Lactobacillus* sp.



a **b**

c

Figure 4. 8 Cell Morphology of isolates FC4-01/1 (a), FC4-01/4 (b) and FC6-01/2 (c) by Gram's staining under light microscope.

Identification of LAB from fermented vegetable and fruit products by biochemical test were found that three isolates were identified as *Lactobacillus* sp. As 30 isolates of lactic acid bacteria were isolates from fermented olives traditionally produced in western Algeria by Kachem(1989) and were identified as *Lactobacillus* sp. Also, Tamminen M. (2004). Identified LAB from fermented cucumbers as a *Lactobacillus* sp.

Because of the problem encountered with the traditional fermentation method of vegetables, relying on the natural microbiota associated with plants, there is an increasing demand for specific starter cultures designed of well-characterized LAB strains. However, a clear identification of species is often rather complicated. Sample phenotypic methods, such as sugar fermentation patterns.

4.9 Identification by 16S-rRNA gene analysis

4.9.1 Amplification of 16S-rRNA gene

Almost full-length 16S-rRNA genes were amplified by PCR using a pair of primer targeting for conserved regions of 16S-rRNA gene in eubacteria, The template DNA were extract from each isolates FC4-01/1, FC4-01/4 and FC6-01/2 were used in 16S-rRNA gene amplification. Results in 1,500 bp of PCR product were amplified from template of each strain when compared with the λ DNA digested with HindIII were used as DNA marker size 23130, 9416, 6557, 4316, 2322, 2027, 564 and 125 bp. (data shown in Fig 4.9)

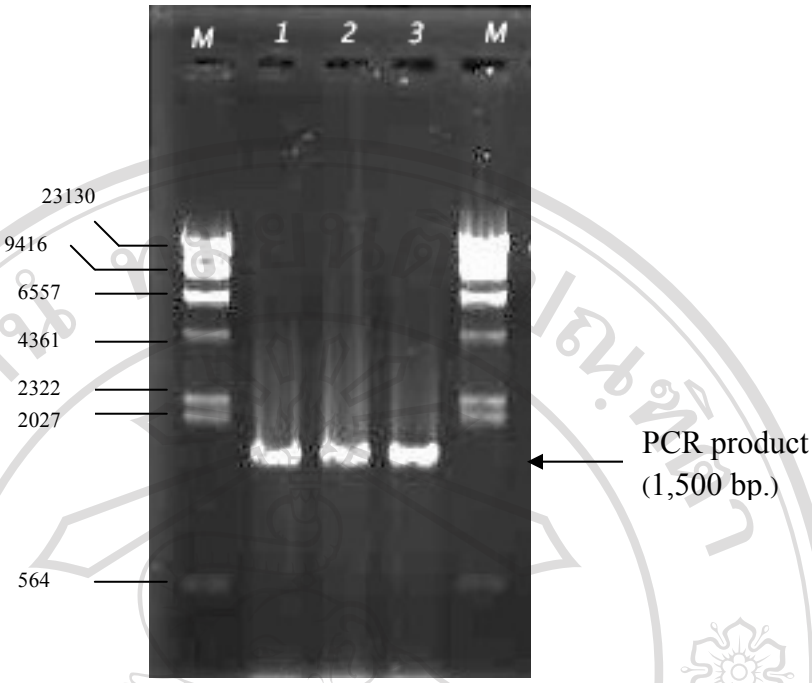


Figure 4.9 Agarose gel electrophoresis of DNA Marker (lane M) and plasmid DNA from isolate FC4-01/1(lane 1), FC4-01/4 (lane 2) and FC6-01/2 (lane 3)

4.9.2 16s-rRNA Gene analysis

To examine the phylogenetic three of 3 isolates, the partial nucleotide sequences of the 16S-rRNA gene were used. The nucleotide sequences were registered in the DDBJ/GeneBank nucleotide databases under the follwing accession number; EU835754-EU835756. The sequences were aligned and compared with corresponding ones retrieved from the DDBJ/GeneBank nucleotide databases. The phylogenetic tree was drawn by tree view of Win 32 program (Figure 4.10)

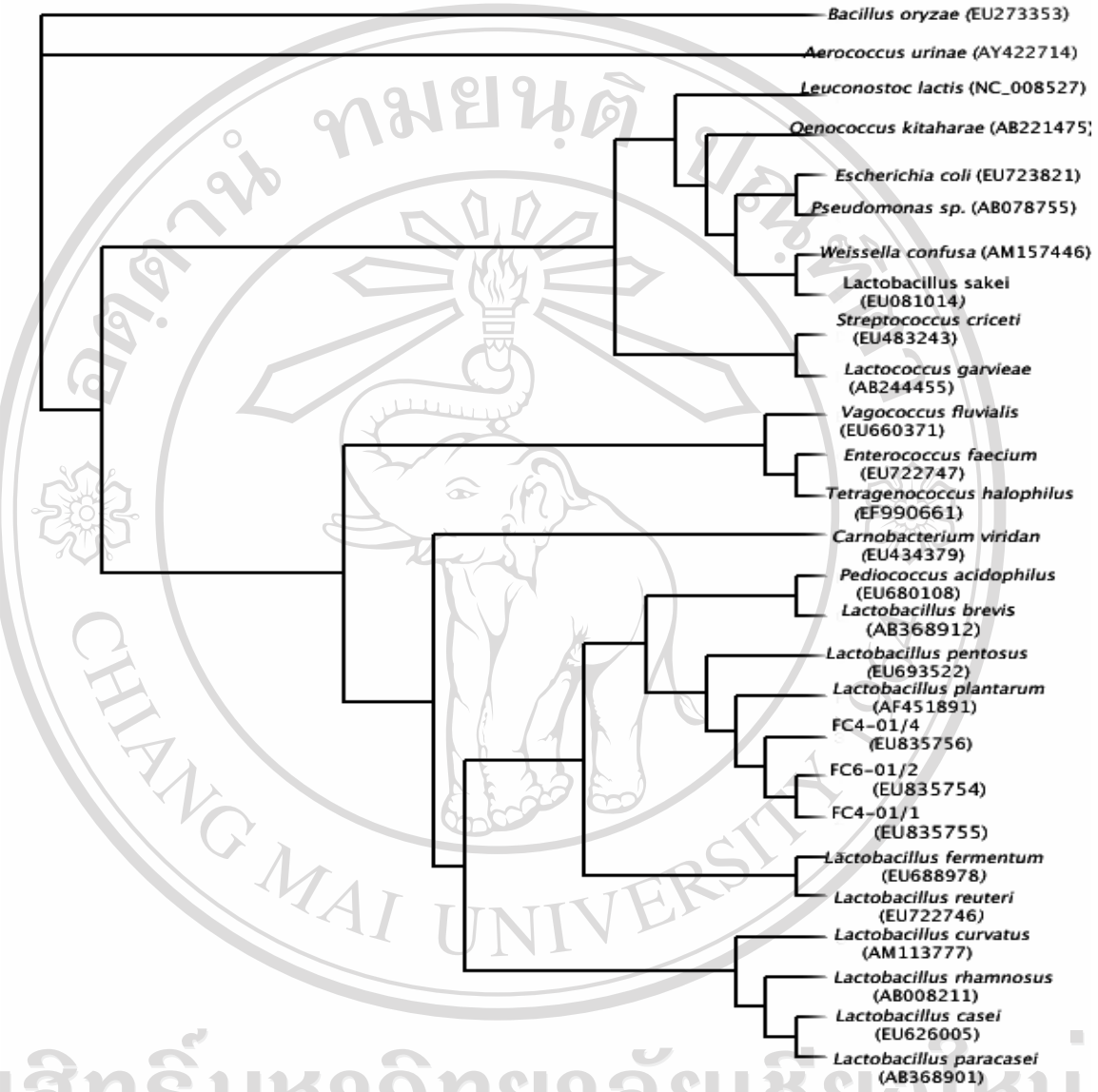


Figure 4.10 Phylogenetic tree of isolates FC6-01/2 (EU835754), FC4-01/1 (EU835755) and FC4-01/4 (EU835756). Based on the partial sequence of 16S-rRNA genes. Numbers in parenthesis are the accession numbers of 16S-rRNA gene sequences in nucleotide databases.

Three isolates were identified as of *Lactobacillus* sp. by biochemical characteristics. The 16S-rRNA sequence analysis of 3 isolates found that isolate FC6-01/2 showed 100% similar to *Lactobacillus plantarum* while isolate FC4-01/1 and isolate FC4-01/4 showed 99% similar to *L plantarum*. Which correspond to report off Karovicava (2003) that lactic acid bacteria from fermented vegetable (pickled of cabbage or Pakguadong) in Thailand produced by *Lactobacillus plantarum*. Many paper report *L. plantarum* produced bacteriocin such as Plantaricin SIK-83, Plantaricin A, Lactolin, Plantaricin B.