CHAPTER III

ISOLATION AND SCREENING OF LACTIC ACID BACTERIA FROM DIFFERENT FOOD SOURCES WITH MILK CLOTTING ACTIVITY

1.1 INTRODUCTION

Lactic acid bacteria refer to a large group of beneficial bacteria that have similar properties and produce lactic acid as an end product of the fermentation process. They are widespread in nature and are found in our digestive systems. Although they are best known for their role in the preparation of fermented food, LABs produce proteolytic enzymes and many of the investigations focused on the degradation of milk proteins. The ability to produce extracellular proteinases having MCE is an important feature of LAB in biochemical. Uses of animal rennet became limited for religious reasons. Most Muslims considered that cheese that is produced by an enzyme which comes from an animal slaughtered on the non- Islamic way is not halal (Nagodawithana & Reed, 1993). In fact, LABs also possess proteolytic enzymes that widely used for produce bioactive compound such as for antimicrobial activity (Belal et al., 2012), antifungal activity, bio-preservation (Gobbettia et al., 2002) and antioxidant activity (Osuntoki & Korie., 2010). The proteolytic behavior of LAB on casein varies according to the bacterial species and environmental conditions (Argyle et al., 1976; Giori et al., 1985; Nour et al., 2014).

Research on production of MCEs from microbial and plant sourcess are reported by several researchers. For example MCE were detected present in *A. niger* (Moosavi-Nasab et al, 2010) and *R. miehei* N (Reps et al., 2006) and *Thermomucor indicae-seudaticae* (Merheb et al., 2010) and *A. oryzae* (Shata, 2005). Furthermore MCE from plants such as *Bromelia hieronymi* (Mariela et al., 2010) and *Solanum dubium* (Isam et al., 2009) and artichoke flowers *Cynara scolymus* (Soledad et al., 2007). Attempts to study the production of MCE from bacteria have focused on *B. subtilis* natto (Shieh et al., 2009) and *B. sphaericus* (El-Bendary et al., 2007), *Streptomycetes* such as *Streptomyces* sp. (Kathiresan & Manivannan, 2007) and *S. clavuligerus* (Keila et al., 2001). LAB is the predominant microbial group in fermented food and is important because of the key role it plays in fermentation processes. Makarova et al, (2006) reported that LAB can be sub-classified into seven groups based on fermented functional properties that include *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc* and *Oenococcus*. Limited work has been reported on the potential of using LAB as a source of MCE except those reported by Hebert et al. (2001) on *L. helveticus* and Sato et al. (2004) on *E. faecalis* TUA2495L.

Characterization of LAB from different sources can be identified by combining the phenotypic and genotypic methods (Hebert et al., 2001). Genotypic identification has been used as substitute or complement to the established phenotypic methods (Tang et al., 1998). Molecular methods have proven to have more specific than the phenotypic methods by distinguishing the differences between the two closely related species (Busch & Nitschko 1999). The phenotypic identification of LAB by using API 50 CHL Kit and genotype-based methods such as 16S rDNA has been extensively

reported (Mohd Adnan et al., 2007; Abd El Gawad et al., 2010; Ali Baradran et al., 2012). Therefore, the purpose of this study was to evaluate proteolytic activity and milk clotting activity of enzyme preparation from LAB isolated from food sources for possible application in dairy products.

1.2 MATERIAS AND METHODS

1.2.1 Isolation of Lactic Acid Bacteria

Lactic acid bacteria were isolated from different sources (Malaysian fermented food such as Budu, Belacan, Pekasam, fermented cocoa, fermented buffalo milk, and yoghurt also from different fruit obtained from local markets and soil). A total of 10 g of sample was homogenized in peptone water (Oxoid) using stomacher (Stomacher R 400 Circular Seward) for 2 min. For fermented fish (Pekasam) and fermented shrimp (Belacan) samples were diluted in 6.5% (w/v) NaCl. The samples were appropriately diluted and spread plated on modified MRS agar (De Man-Rogosa). The plates were incubated anaerobically at 37°C for 48 h.

1.2.2 Gram Staining

The method as described in food microbiology (Ahmed & Carolyn, 2004). A sterile wire loop was used to slightly touch a colony of each isolate and emulsified in a drop of distilled water on a clean slide to make a thin smear. This was air dried, then heat fixed. The smear was flooded with crystal violet stain and left for 30 sec, after rising under a gently running tap. Two drops of Gram's iodine solution was then added to

act as a mordant. This was left for 30 sec and washed off under a gently running tap 95% ethanol was used to decolorize the smear for 10 sec. or until it appeared free of crystal violet stain. It was then rinsed under a running tap. Counter staining was done with 2 drops safranin for 30 sec and then rinsed off under a running tap. The smear was blotted dry and left to air-dry before viewing microscopically under oil immersion objective lens.

1.2.3 Catalase Test

The method as described in food microbiology methods. A sterile loop was used to touch a colony of overnight isolates and transferred onto a clean glass slide. Then a drop of 3% hydrogen peroxide (H_2O_2) was then added and a reaction was observed. Production of gas bubbles as white froth indicates a catalase positive reaction while the absence of the troth indicates a negative reaction. All the isolates were maintained in 15% glycerol stock at -20ºC.

1.2.4 Screening of Lactic Acid Bacteria for Milk Clotting Activity

Lactic acid bacteria were sub-cultured in MRS agar was anaerobically incubated at 37ºC for 24 h, anaerobically. Each strain was inoculated into a test tube containing 5 ml of litmus milk medium (Oxoid) and cultured statically at 30ºC for 7 d. Strains which formed good clot of casein with clear and little whey were selected for further study.

1.2.5 Characterization of Lactic Acid Bacteria with Proteolytic Activity

3.2.5.1 Bacterial Growth in Different Concentrations of Sodium Chloride

Different concentrations 1.5%, 3.5%, 6.5%, and 7.5% of NaCl were dissolved in 100 ml of MRS broth (Oxoid), respectively, dispensed into screw cap bottles and sterilized. The bottles were inoculated with LAB and incubated at 37ºC for 48 h. Turbidity of the medium indicates growth, while un-inoculated bottles served as control.

3.2.5.2 Bacterial Growth at Different Temperature

MRS broth was sterilized and inoculated with the LAB isolates and incubated at 10, 37 and 45ºC anaerobically respectively for 72 h. Turbidity of the medium was compared with un-inoculated control bottles and served as indicator of growth by the isolates.

3.2.6 Detection of Extracellular Proteinase

The production of extracellular proteinase was detected following the method described by Pailin et al. (2001) and Aween et al. (2012) with some modification as follows: 25g of skim milk was reconstituted with 250 ml of distilled water, stirred thoroughly and autoclaved at 110ºC for 10 min. Agar No. 3 (Oxoid) 500 ml of 2.5% (w/v) was sterilized at 121 ºC for 20 min then mixed with skim milk and poured into petri dish. Lactic acid bacteria was spot inoculated on prepared skim milk agar media

then incubated at 37ºC for 48 h in an anaerobic jar followed by cooling room at 4ºC for 3 d. Protein digestion was observed by the production of clear haloes surrounding the colonies.

3.2.7 Identification of Lactic Acid Bacteria

3.2.7.1 Carbohydrate Fermentation Profile using API50 CH of Selected Lactic Acid Bacteria Isolates

The API 50 CHL strips (API systems, Biomerieux, France) consisting of 50 microtubes were used to identify the LAB isolates based on carbohydrate fermentation profile. Overnight bacteria cultures were incubated according to the manufacturer's instructions and the bacterial suspension was prepared with the turbidity equivalent to McFarland 2 standard. The strips of the API 50 CHL test were filled with the inoculated API suspension and overlaid with mineral oil. The inoculated strips were incubated at 37ºC and the reactions were observed after 24 and 48 h of incubations. The results were analyzed by API software version (BioMerieux).

3.2.7.2 Identification of Lactic Acid Bacteria using PCR

3.2.7.2.1 DNA Extraction of Lactic Acid Bacteria Isolates

Genomic DNA of LAB isolates was extracted using (Genomic DNA Extraction Mini Kit, Yeastern Biotech CO., LTD, Taiwan) as with manifesting instructions with some modifications. Briefly, 1500 µl of overnight culture was harvested by centrifuging at

14,000 x *g* for 1 min using (Eppendorf centrifuge 5804 R) and the supernatant was discarded. The cell pellet was re-suspended in 200 µl of lysozyme and then incubated at room temperature 25ºC for 10 min with shaking the tubes every 2 to 3 min. 200 µl of GB buffer was added to the sample, and mixed by vortexing for 5 sec. The LAB samples were incubated at 37ºC for 20 min until the sample lysate was clear. During incubation, the sample tubes were upturned every 3 mins. At that time, elution buffer (200 µl per sample) incubated at 60 ºC (for DNA elution). Following that, a volume of 200 µl of the ethanol was added to each sample list and mixed immediately by vortexing for 10 sec. GD column was placed on a 2 ml collection tube. Then all the mixture from the previous step was applied to the GD column, and was then centrifuged at 14000 x *g* for 2 min. The flow-through was discarded and the GD column placed in a new 2 ml collection tube. 400 µl of W1 buffer (ethanol added) into the GD column, and then centrifuged at 14000 x *g* for 30 sec. the flow-through was discarded and the GD column placed back in the 2 ml collection tube, the wash step was repeated with 600 µl of wash buffer (ethanol added) with centrifuge at 14000 x *g* for 30 Sec. the flow-through was discarded and the GD column placed back in the 2 ml collection tube, then centrifuged at 14000 x *g* for 3 min to dry the column matrix. The dried GD column was transferred into a clean 1.5 ml micro-centrifuge tube, and was then preheated with 100 µl elution buffer was added in the centre of the column matrix. Stand for 3 to 5 min until elution buffer absorbed by the matrix. The purified DNA was eluted by centrifuging at 14000 x *g* for 30 sec. Finally, the DNA sample was kept at -20ºC for analysis.

3.2.7.2.2 PCR Amplification of 16S rDNA gene Sequence of Lactic Acid Bacteria Isolates

The extracted DNAs were used directly in PCR reactions to amplify the 16S rDNA gene from LAB. The 16S rDNA region was amplified by using primers (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3). The PCR reaction mixture contained 5 µl of 1X reaction buffer ($NgCL_2$) (Yeastren Biotech CO., LTD. Taiwan), 1 µl of 0.2 mM dNTPs mix (Yeastren Biotech CO., LTD. Taiwan), 1 µl of 10 µM 16S forward and 1 µl of 10 µl 16S reverse primers, 1 µl of template DNA, and 0.5 µl of (1.25 U/ µl) YEAtaq DNA polymerase (Yeastren Biotech CO., LTD. Taiwan). A negative control without DNA template was included in parallel. Each sample toped up with 40.5µl ddH_2O until 50 µl. The PCR were as follows: (LID 105ºC), initial at 95ºC for 3 min, denaturation at 95°C for 30 sec. annealing at 57ºC for 15 sec. and extension at 73ºC for 1 min, with 35 cycles for each step. Final elongation was at 73ºC for 5 min; and then held at 4°C. From each amplification mixture 10 µl were mixed with 2 µl of 6X DNA loading dye (Fermentans), and then subjected to electrophoresis in 1.0% (w/v) agarose gels (Conda S.A, Spain) in 1x TAE buffer (Bio Basic Canada INC, Canada) for 40 min and 80 V. 6 µl of 1Kb DNA ladder (250 to 10000 bp) from (1^{st} Base, Malaysia) was used as standard. After electrophoresis the gel was stained in ethidium bromide (Merck, Germany) for 30 min and then washed with distil water for another 30 min. The gel was visualized and photographed with UV transilluminator (BIORAD).

3.2.7.2.3 PCR Product Cleanup

The PCR product was purified using PCR clean up kit from (Yeastern Biotech, Taiwan). Initially 40 µl of the PCR product added into a micro-centrifuge tube with 200µl of DF buffer and mixed by vortexing the tube. The sample was applied into DF column with collection tube, and then centrifuged at 6000 x *g* for 30sec. The flow-through was discarded and the DF column placed back in the collection tube. The centrifugation was repeated at 14000 x *g* for 2 min.the dried DF column was transferred to a new micro-centrifuge tube. 15µl of the elution buffer was added in the centre of the DF column matrix, stand for 2 min until the elution buffer absorbed by the matrix. Purified DNA was eluted at 14000 x *g* for 2 min.

3.2.7.2.4 PCR Product Sequencing

The partial 16S-S DNA and 16 S-R DNA PCR product of isolates was sequenced by sending to 1st Base, Malaysia and sequences were compared (analyzed) by the BLAST program on the NCBI website databases (http://www.ncbi.nlm.nih.gov/). (Gen- Bank).

3.2.8 Production Media for Milk Clotting Enzyme

The enzyme production media consisted of 10g tryptone soya, 10g glucose, 2g $CH_3COONa \cdot 3H_2O$, 0.2g $MgSO_4 \cdot 7H_2O$, 0.01g $MnSO \cdot 4H_2O$, 0.01g $FeSO_4 \cdot 7H2O$, 0.01g NaCl , dissolved in 1000 ml of distilled water and adjust the pH to 6.8 using NaOH (0.1 M). One ml of the 24 h pre-cultured broth of LAB was inoculated into

Erlenmeyer flask containing 100 ml of the enzyme production medium with or without 1% $CaCO_3$ previously sterilized by autoclaving at 121°C for 15 min. The enzyme production, media with $CaCO_3$ was gently shaken in a shaker incubator (SASTEC- MODEL-ST-200R) at 150 kept at 30°C for 48 h, followed by centrifuging at 9,000 *x* g for 20 min at 4°C. The supernatant was collected and filtrated regenerated cellulose membrane filter (filter size 0.22 µ (SARTORIUS STEDIM BIOTECH.) for MCA and PA measurement, and determination of MCA/ PA ratio (Sato et al., 2004).

3.2.9 Milk Clotting Enzyme Assay

Enzyme assay was carried out according to the method described by Arima et al. (1968) and Sato et al. (2004). Milk clotting activity was expressed as Soxhlet units (SU) in which 1 SU is defined as the amount of enzyme that clots 1 ml of a solution containing 0.1 g skim milk powder and 0.00111 g $CaCl_2$ in 40 min at 35°C. Under this assay condition, 400 units of activity (SU) is defined as the amount of enzyme that clot the milk solution in 1 min. Soxhlet unit is defined as the amount of enzyme (400 unit of activity) which clotted milk solution in 1 min.

Skim milk (10% w/v, Oxoid) containing 10 mM $CaCl_2$ was used as a substrate, pre-incubated at 35°C for 5 min. Enzyme extract 0.5 ml was added to 5 ml of substrate solution, mixed well and incubated at 35°C. The time *t* (sec), the time period starting from the addition of the enzyme to the first appearance of clots of milk solution was recorded. MCA was calculated using the following formula.

$$SU = 2400/T \; x \; S/E$$ whereas,

T = clotting time (sec), S = substrate solution (ml), E = enzyme solution (ml)

3.2.10 Proteolytic Activity of Milk Clotting Enzyme

The method described by Sato et al. (2004) was used to determine the proteolytic activity of the cell free enzyme production media. Hammerstein casein 1% was dissolved in 0.1 M Tris – Hcl buffer pH 7.5 as substrate, and 5 ml of this substrate solution was incubated with 1ml of crude enzyme solution at 45°C for 30 min, then the enzyme reaction was stopped with 5 ml of trichloroacetic acid mixture (0.11 M CCl_3COOH, 0.22 M CH_3COONa and 0.33 M CH3COOH). After incubation for 30 min the mixture was filtered using Whatman filter paper No.1, and 2 ml of the filtrate was added to 5 ml of 0.55M Na_2CO_3 and 1 ml of Folin's reagent (diluted 1:3).

The reaction mixture was held at 30°C for 30 min and the optical density at 660 nm was measured using UV-Visible Spectrophotometer (4001/4, Thermo Spectronic, USA). One unit of proteolytic activity is defined as the amount of enzyme which released 1 µg of amino acid expressed as the tyrosine concentration per min under the above condition. The change in absorbance in test samples was determined by calculating the difference between test samples absorbance and absorbance of test blank. Inserting the absorbance value for one of the test samples into the slope equation, micromoles of tyrosine liberated during the proteolytic reaction was determined as follows.

$$\frac{\textit{Units/ml enzyme} = \textit{(u mole tyrosine equivalents released) x (V1)}}{\textit{(V2) (T) (V3)}}$$

Where:-

V1 = total volume (in ml) of assay

T = time of assay in min as per the unit defined

V2 = volume of enzyme (in ml) used

V3 = volume (in ml) used in colorimetric determination

3.2.11 **Statistical Analysis**

A one way analysis of variance (ANOVA) using MINITAB version 16 was carried out and the P value was set 0.05 ($P<0.05$) for significant differences. Tukey's pairwise comparison was used to compare mean differences on the effects of different LABs isolates and their interactions on clotting time, milk clotting activity, proteolytic activity and ratio of milk clotting activity to proteolytic activity.

3.3 RESULTS

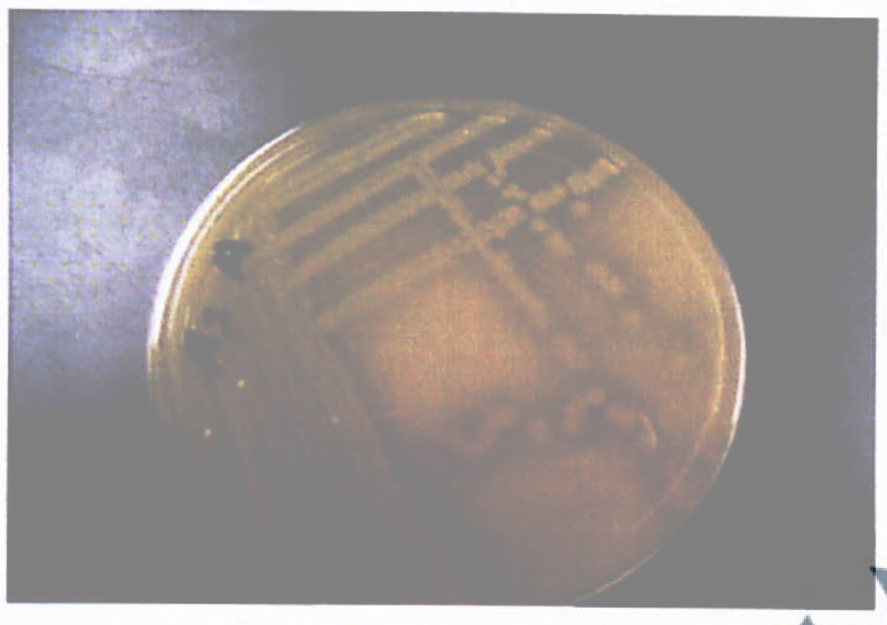
3.3.1 Isolation and Characterization of Lactic Acid Bacteria

A total of 135 LAB was isolated from different sources of fermented food. The isolates were classified based on their morphology and biochemical characters all colonies exhibiting clear halos on MRS agar with 0.8% $CaCO_3$ the isolates were Gram positive and catalase negative (Table 1).

Table1: Characterization of LAB Isolates

Isolate code	Isolate sources	Number of isolates	Gram staining	Catalase test	Morphology
FFB1	Budu1	7	+	-	Cocci
	Budu1	4	+	-	Short rod
FFA1	Budu2	3	+	-	Cocci
	Budu2	6	+	-	Short rod
	Budu2	4	+	-	Bacilli
	Belacan	5	+	-	Cocci
SH	Belacan	4	+	-	Short rod
	Belacan	3	+	-	Bacilli
CF1	Pekasam	7	+	-	Cocci
	Pekasam	5	+	-	Short rod
	Pekasam	3	+	-	Bacilli
CO1	Fermented cocoa	2	+	-	Cocci
	Fermented cocoa	4	+	-	Short rod
	Fermented cocoa	4	+	-	Bacilli
YO1	Yoghurt	2	+	-	Cocci
	Yoghurt	5	+	-	Short rod
	Yoghurt	4	+	-	Bacilli
AS.A	Ginger	3	+	-	Cocci
	Ginger	2	+	-	Short rod
	Ginger	5	+	-	Bacilli
GOO7	Guava	2	+	-	Cocci
	Guava	6	+	-	Short rod
	Guava	5	+	-	Bacilli
H6M	Buffalo milk	8	+	-	Cocci
	Buffalo milk	4	+	-	Short rod
	Buffalo milk	9	+	-	Bacilli
SO	Soil	4	+	-	Cocci
	Soil	6	+	-	Short rod
	Soil	5	+	-	Bacilli
Total		135			

Figure 3: Lactic acid bacteria on MRS agar with $CaCO_3$



3.3.2 The Effect of Temperature and Sodium chloride on Growth of Lactic Acid Bacteria Isolates

All the isolates grew at 10, 37 and 45°C, but growth of FFA1, SH, SO was not observed at 10°C (Table 3). Growth at 1.5% NaCl was observed for all LAB isolates, however, their growth was inhibited at higher NaCl concentrations except FFA, SH and CF1 (Table 2) which were isolated from Malaysian fermented food (Belacan and Pekasam).

Table 2: The Effect of Temperature and NaCl on Growth of LAB Isolates*

Isolate code	Growth temperature (°C)			Growth in NaCl (%)				
	10	37	45	1.5	3.5	5.5	6.5	7.5
FFA1	-	+	+	+	+	+	+	+
SH	-	+	+	+	+	+	+	+
CF1	-	+	+	+	+	+	+	+
CO1	+	+	+	+	-	-	-	-
YO1	+	+	+	+	-	-	-	-
AS.A	+	+	+	+	-	-	-	-
GOO7	+	+	+	+	-	-	-	-
H6M	+	+	+	+	-	-	-	-
SO	-	+	+	+	-	-	-	-

*(+) growth (-) No growth

3.3.3 Detection of Extracellular Proteinase and Screening for Milk Clotting Activity by Litmus Milk Test

Ten of 135 LAB that produces extracellular proteinase enzyme on skim milk agar were evaluated for their ability to clot milk based on litmus milk test. Result found that, the isolates SH and CF1 isolates milk clot that was large, soft with very little amount of whey (Table 3).

Table 3: Proteolytic Activity on Skim Milk Agar and Litmus Milk Test

Isolate code	Casein hydrolysis	Litmus milk test
FFB1	+	Yellow weak curd
FFA1	+	Yellow weak curd
SH	+	Purple soft curd
CF1	+	Purple weak curd
CO1	+	No change
YO1	+	No change
AS.A	+	Pink weak curd
GOO7	+	No change
H6M	+	Pink weak curd
SO	+	No change

Figure 4: Plate Showing Proteolytic Activity

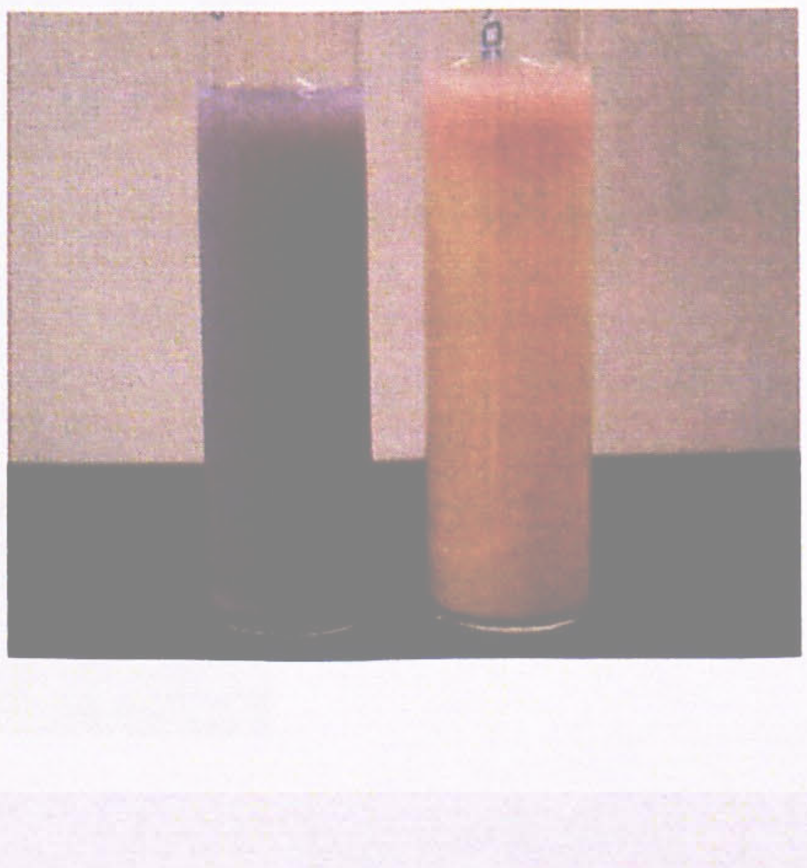


Figure 5: Screening of LAB for MCA by Litmus Milk Test

3.3.4 Identification of Lactic Acid Bacteria Isolates

3.3.4.1 Identification of Lactic Acid Bacteria Isolates by (API 50 CH KIT)

The results in Table 4 show that the identification of the isolates was performed through the fermentation of the carbohydrates in an API CH 50 kit (BioMeurix – France).

Table 4: Isolates Identification using the API 50 CHL kit

Source of isolates	Code	Strain	%ID	T
Belacan	SH	*Pediococcus acdilactici*	99.9	0.80
Pekasam	CF1	*Lactobacillus lactis cremoris*	93.7	0.66

3.3.4.2 Identification of Lactic Acid Bacteria by (16s rDNA gene Sequences)

Result of sequencing analysis of purified PCR showed that the isolates SH and CF1were isolated from belacan and pekasam identified as *Pediococcus acdilactici* and *Lactobacillus paracasei* (Table 5).

Figure 6: 16S.S: (5-AGAGTTTGATCCTGGCTC-3) and 16S.R: (5-CGGGAACGTATTCACCG-3), (1) Lane1kb, DNA ladder; (2) SH (3) CF1.

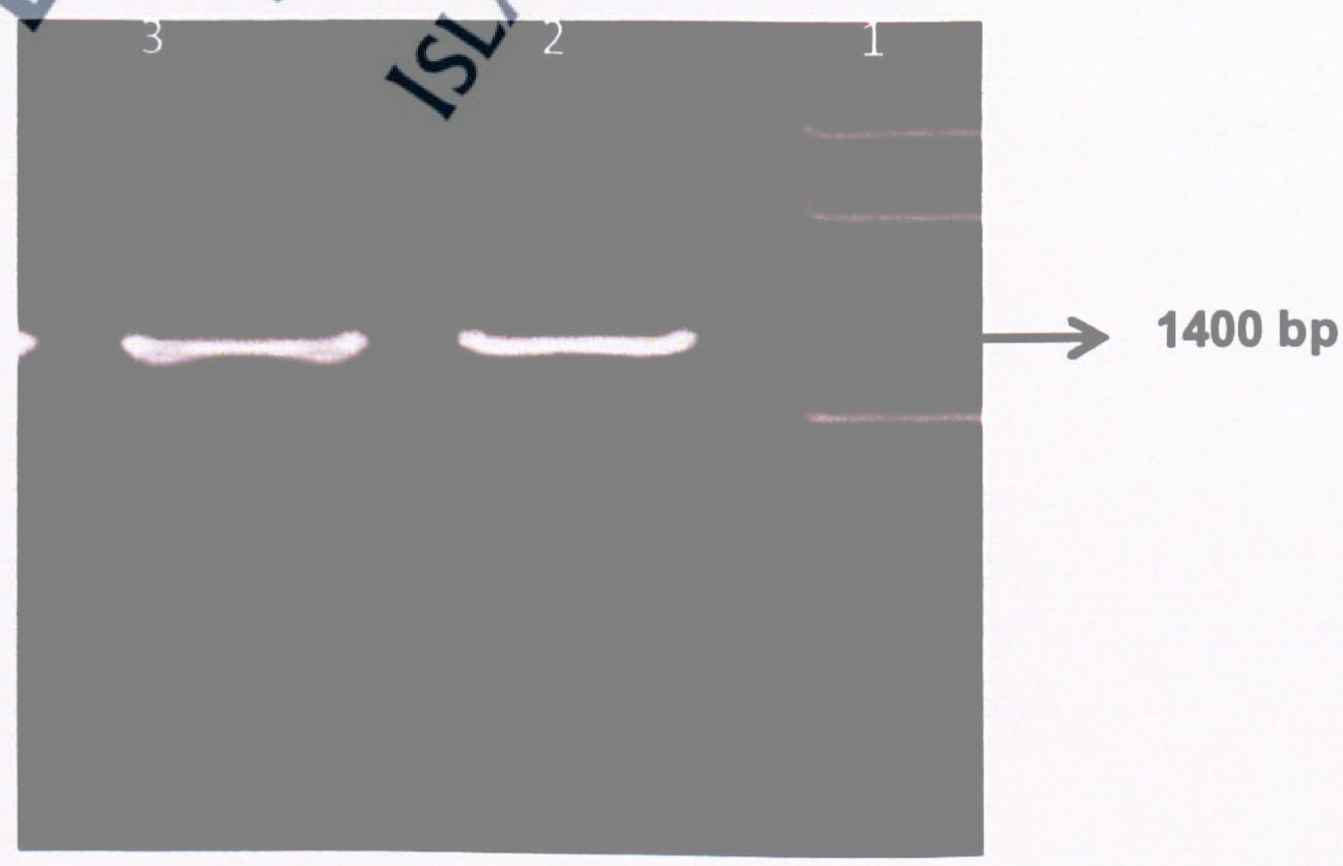


Table 5: Lactic Acid Bacteria Identified using 16S rDNA Sequencing

Code of isolates	Identification	% identification.
SH	*Pediococcus acidilactici*	99.7%
C.F1	*Lactobacillus paracasei*	99.0%

3.3.5 Milk clotting Activity and Proteolytic Activity of crud enzyme of Lactic Acid Bacteria Isolates

Ten from 135 LAB isolated from different food sources exhibited extracellular proteolytic enzyme on skim milk agar were further evaluated for their MCA. It was observed that only six of the ten isolates namely SH, CF1, FFA, FFB, H6M, and ASA showed MCA (Table 6). The isolates SH and CF1 showed lowest milk clotting time (72 and 82 Sec, respectively), and the highest MCA (50 SU/ml and 43 SU/ml, respectively) also low PA (1.9 ml and 3.5 ml, respectively). The ratio of MCA/PA of SH and CF1 was higher than other isolates. Other isolates, FFA, FFB, AS.A, and H6M showed longer clotting time (104, 109, 96, and 118 sec. respectively and PA (3.6 ml, 3.8 ml, 3.8 ml and 3.5 ml, respectively. MCA of these LAB isolates was low (34 SU/ml, 32 SU/ml, 32 SU/ml, and 30 SU\ml, respectively). Higher MCA/PA ratio is desirable for milk clotting enzyme and it generally indicates the restricted degradation of casein. Therefore, SH and CF1 isolates were selected for further experiments because of its high MCA/PA ratio 26.3 and 12.6, respectively.

Table 6: MCA, PA of Crude Enzyme of LAB Isolated from Different Sources

Code of isolates	Clotting time(s)	MCA(SU/ml)	PA(U/ml)	MCA/PA
AS25	0.00^{g}	0.00^{d}	3.20^{ab}	0.00^{c}
CF1	82.0^{e}	44.0^{b}	3.50^{ab}	12.6^{b}
SH	72.0^{f}	50.0^{a}	1.90^{c}	26.3^{a}
Co1	0.00^{g}	0.00^{d}	3.10^{ab}	0.00^{c}
FFA	104.0^{c}	34.0^{c}	3.60^{ab}	9.40^{b}
FFB	109.0^{b}	32.0^{c}	3.80^{ab}	8.40^{b}
SO	0.00^{g}	0.00^{d}	3.20^{ab}	0.00^{c}
GOO7	0.00^{g}	0.00^{d}	3.00^{b}	0.00^{c}
H6M	96.0^{d}	32.0^{c}	3.90^{a}	8.40^{b}
ASA	118.0^{a}	30.0^{c}	3.50^{ab}	8.50^{b}

*(a-g) Means in the same column followed by different letters were significantly different ($P<0.05$).

3.4 sDISCUSSION

Lactic acid bacteria play an important role in successful fermentation of foods such as milk, fish, meat and vegetables. The production of organic acids and other compounds contribute to the antimicrobial activity of LAB and thus make fermented food safe (Saranraj et al., 2013). LABs also influence desirable flavor and texture changes in the product (Rhee et al., 2011). In yogurt for example, addition of LAB starter cultures reduce the pH, resulting in acidification of milk and precipitation of casein and whey protein as well as for flavor development (Jaiswal et al., 2014). In cheese however, rennin is added to coagulate milk casein and non-starters LAB are added for flavor and modifying the texture of cheese during aging (McSweeney & Sousa 2000). Proteolysis has been widely used as a basis for classification of cheese (Sousa & McSweeney, 2001). In present study ten of 135 LAB were able to produce extracellular proteinase as indicated by clear zone surrounding the colonies on skim

milk agar plate. Six of the ten isolates namely SH, CF1, FFA, FFB, H6M, and AS.A have the ability to produce MCE with milk clotting activity. Thus the MCE obtained from strain SH produced high milk clotting activity and, ratio of MCA/PA was (50 SU/ml) and 26.3, respectively compared with other strains (Table 6). The MCA obtained from SH strain was higher than that *E. faecalis* 2495L, *E. faecalis* IAM10065 (28.3 SU/ml) and *E. faecalis* 156 (22.7 SU/ml) with MCA/PA 3.8 as reported by Sato et al. (2004). However, Merheb et al. (2010) reported that MCA and MCA/PA obtained from *T. indicae-seudaticae* N31 were (160.3 SU/ml) and 76, respectively. Higher MCA/PA ratio is desirable for milk clotting enzyme and it generally indicates the restricted degradation of casein (Sato et al., 2004).

Milk clotting time is affected by the enzyme, concentration of the enzyme, pH, temperature and Ca^{2+} and species of LAB also depending on the proteolytic system of LAB (Smit et al., 2002; Leitner et al., 2006). In this study the time required to coagulate milk for the extracellular enzyme produce by SH isolate was 72 sec. less than the other isolates, but similar to *S. esculentum* 73 sec. as reported by Guiama et al, (2010). LAB with proteolytic activity and MCA were reported by several researchers, for example, *L. helveticus* (Hebert et al., 2001), *L. paracasei* (Haq-ul & Mukhtar, 2006) and *E. faecalis* 2495L, *E. faecalis* IAM10065 and *E. faecalis* 156 (Sato et al. (2004). Milk clotting activities of microorganism have different characteristics and values. Fungi especially *Mucor, Rhizopus, Endothia, Rhizomucor* and *Aspergillus* have been used for production of MCE. Some *Bacillus* spp. notably *B. subtilis, B. cereus, B. licheniformis* (D'Souza & Pereira, 1982; Dutt et al., 2008) and others like *Myxococcus xanthus* strain 422 (Poza et al., 2003) and *Nocardiopsis*

(Cavalcanti et al., 2005) were reported to produce enzyme with MCA. LAB has also been reported as MCE producers such as *L. helveticus* (Hebert et al., 2001). Four from 157 LAB (*E. faecalis* 2495L, *E. faecalis* IAM10065 and *E. faecalis* 156) were isolated from different fermented food produces MCE with milk clotting activity (MCA) (Sato et al., 2004).

The ability to produce extracellular proteinases is a very important feature of LAB. This is because most of LAB isolated from fermented products has multiple amino acid auxotrophy and in order to grow in the protein rich medium they depend on the expression of a complex proteolytic system for the degradation of protein (Kok, 1993; Visser, 1993). In addition, proteinase play significant role in the formation of cheese texture due to protein degradation (Olson, 1990). Increasing demand for dairy product, low availability of rennet has led to the replacement of calf rennin by microbial milk clotting enzymes (Kurutahalli et al., 2010). A crude enzyme from SH and CF1 isolates showed the highest milk-clotting activity and lowest proteolytic activity. In contrast, the other isolate FFB FFA, ASA and H6M showed high proteolytic activity but low MCA. Fungi especially *Mucor, Rhizopus, Endothia, Rhizomucor, and Aspergillus* have been used for production of MCE. Some *Bacillus* sp. notably, *B. subtilis, B. cereus, B. licheniformis* (D'Souza & Pereira 1982; Dutt et al., 2008) and others like *Myxococcus xanthus* strain 422 (Poza et al., 2003) and *Nocardiopsis* (Cavalcanti et al., 2005) were reported to produce an enzyme with MCA.

High proteolytic activity normally leads to lower yield, soft body and bitter taste of milk curd (Chazarra et al., 2007; Vishwanatha et al., 2010). The cheeses obtained by

curdling milk with molds produced rennet, develop bitterness when maturation period is long, this being a constraint in the use of this microbial rennet (Samson et al., 2004). Therefore, the ratio of MCA to PA is used as an indicator for selecting the potential strain.

Many LABs have been isolated from fermented seafood such as belacan, budu, cincaluk, pekasam, trassi, kicap, tauco, tompoyak and sambal belachan (Ohhira et al., 1990). Varying concentrations of salt are added to modify the environment in fermentation of traditional fermented foods. The salt concentration in belacan is 1.7% budu 8.66% and pekasam 1.5% (Ohhira et al., 1990). This will influence the growth requirement of LAB present in these products. It was observed that SH isolated from belacan. LAB has ability to grow in NaCl concentration ranging from 1.5 to 10% salt concentration (Nanasombat et al., 2012). Salt is normally added in fish fermentation. The LAB isolated from pekasam, belacan and budu tolerated 7.5% NaCl compared to other LAB isolates. Papamanoli et al. (2003) reported that seven *L. planetarum* strains isolated from naturally fermented dry sausage have the ability to grow in 6.5, 8.0, and 10% (w/w) of NaCl. Similarly, Cho and Ki, (2006) isolated halo-tolerant strains of *Leuc. mesenteroides* and *Str. salivarius* from Korea fermented food. Jeotgal is made from raw seafood and fish by traditional methods with 6.5% NaCl. The salt tolerance gives them an advantage over other less tolerant species and allows the lactic acid fermenters to begin metabolism, which produces acid, which further inhibits the growth of non-desirable organisms (Oyewole & Isah, 2012). Growth of LAB at specific temperature and NaCl concentrations are used to group between thermophillic cocci and enterococci LAB that grow at 4ºC and 6.5% NaCl are considered as

thermophilic cocci, while those that grow at 10ºC and 45ºC in the presence of 6.5% NaCl were considered as enterococci (El-Soda et al., 2003).

The identification of LAB based on the physicochemical and biochemical characteristics is often considered as unreliable, since different species may have similar morphology and nutritional requirements (Ben-Amor et al., 2007). Therefore, the phenotypic characterization based on carbohydrates fermentation profile may be used as a possible identification, but it may not provide trusty identification. The identification method, genotype-based methods such as 16S rDNA are strong to identify bacteria as a complement or alternative to phenotypic methods (Weisburg et al., 1991). Genotype methods are independent from variation of growth conditions if species-specific primers or probes are available; these offer a very fast way to detect the target organism. It is clear from the above discussion that a careful consideration of numerous factors has to be made for the correct identification of specific species of LAB (Gyu Sung et al., 2006; Mohd-Adnan et al., 2007; Abd El Gawad, el al., 2010; Ali Baradran et al., 2012).

Based on the biochemical and API CHL 50 Kit, the isolates of SH and CF1 showed 99.9 % and 93% homology to the *Pediococcus acidilactici*, and *Lactobacillus lactis cremoris*, respectively. However, with 16S rDNA sequence analysis methods, the isolated LAB was 99.3% and 99% was identified be *Pediococcus acidilactici* and *Lactobacillus paracasei*. The biochemical and API CHL50 Kit compared to 16S rRNA, the result of this study using molecular methods very important for typing newly isolated microorganisms.

3.5 CONCLUSION

Dairy industry seeks for novel enzyme sources, and microbial coagulants have several advantages over animal and plant source. Lactic acid bacteria are used in the production of a wide range of dairy products and have influences on flavor development and ripening. In this study a total of 135 LABs isolates were isolated from different Malaysian fermented foods were screened for produce extracellular enzyme based on skim milk agar. Two LBAs isolated from belacan, and pekasam were identified by (API 50CHL) and confirmed by the sequence analysis of 16S rDNA gene as *Pediococcus acidilactici* SH, and *Lactobacillus paracasei* CF1. The results show the properties of the extracellular enzyme produce by (SH) and (CF1) isolates that indicate the possibility of the use of these extracellular enzymes in the dairy manufacture.