คุณลักษณะของเอนไซม์ที่สามารถตกตะกอนนมจากแบคทีเรียที่คัดแยกได้จากกระบวนการหมักน้ำปลา

นางสาวศศิธร แก้วเผือก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHARACTERIZATION OF MILK-CLOTTING ENZYMES FROM BACTERIA ISOLATED FROM FISH SAUCE FERMENTATION

Sasitorn Kaewphuak

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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Suranaree University of Technology

Academic Year 2011

CHARACTERIZATION OF MILK-CLOTTING ENZYMES FROM BACTERIA ISOLATED FROM FISH SAUCE FERMENTATION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ศศิธร แก้วเผือก : คุณลักษณะของเอนไซม์ที่สามารถตกตะกอนนมจากแบคทีเรียที่คัดแยกได้จากกระบวนการหมักน้ำปลา (CHARACTERIZATION OF MILK-CLOTTING ENZYMES FROM BACTERIA ISOLATED FROM FISH SAUCE FERMENTATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 114 หน้า.

แบคทีเรียชอบเค็มปานกลางที่คัดแยกได้จากกระบวนการหมักน้ำปลาผลิตเอนไซม์ที่หลั่งออกมานอกเซลล์ที่แสดงกิจกรรมการตกตะกอนนม วัตถุประสงค์ของงานวิจัยนี้คือการคัดเลือกและระบุชนิดของแบคทีเรียที่คัดแยกได้จากกระบวนการหมักน้ำปลาที่แสดงความสามารถในการตกตะกอนนมสูงสุดและศึกษาถึงสมบัติบางประการของเอนไซม์ตกตะกอนนม จากแบคทีเรีย 19 ไอโซเลท พบว่าไอโซเลท SK1-3-7 SK1-1-8 และ SK39 แสดงอัตราส่วนระหว่างกิจกรรมของเอนไซม์ที่ตกตะกอนนมต่อกิจกรรมการย่อยสลายโปรตีนสูงที่สุด การระบุชนิดของแบคทีเรียโดยอาศัยลักษณะทางสัณฐานวิทยาและสรีรวิทยา พบว่าทั้ง 3 ไอโซเลท เป็นแบคทีเรียแกรมบวก รูปร่างเซลล์เป็นท่อนที่สร้างสปอร์และเป็นแบคทีเรียชอบเกลือปานกลาง ทุกไอโซเลทเจริญในสภาวะที่มีเกลือโซเดียมคลอไรด์ 0-20% และเจริญได้ดีในสภาวะที่มีเกลือโซเดียมคลอไรด์ 5-15% ไอโซเลท SK39 และ SK1-1-8 เจริญที่พีเอช 6-10 ในขณะที่ไอโซเลท SK1-3-7 เจริญที่พีเอช 6-11 ทั้ง 3 ไอโซเลทเจริญได้ที่อุณหภูมิ 10-45 องศาเซลเซียส มีองค์ประกอบของควิโนน (Quinone) ชนิด เมนาควิโนน-7 (MK-7) เป็นองค์ประกอบหลัก และมี anteiso-$C_{15:0}$ anteiso-$C_{17:0}$ เป็นกรดไขมันหลักของเซลล์ จากผลการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rRNA gene พบว่าแบคทีเรียที่คัดเลือกจัดอยู่ในสกุล *Virgibacillus* โดย SK1-3-7 SK1-1-8 และ SK39 มีความเหมือนของลำดับนิวคลีโอไทด์กับ *V. halodenitrificans* 99.4 99.6 และ 99.6% ตามลำดับ ผลของการวิเคราะห์ DNA-DNA relatedness ระหว่างไอโซเลทเหล่านี้กับ *V. halodenitrificans* JCM12304^T มีค่าความเหมือนเท่ากับ 38.96-45.85% ดังนั้นแบคทีเรียไอโซเลท SK1-3-7 SK1-1-8 และ SK39 เป็นแบคทีเรียชนิดใหม่ในสกุล *Virgibacillus*

เอนไซม์ตกตะกอนนมจาก *Virgibacillus* sp. SK1-3-7 ที่ผ่านการทำให้บริสุทธิ์บางส่วนประกอบด้วยโปรตีนที่มีน้ำหนักโมเลกุล 20 และ 36 กิโลดาลตัน เมื่อวิเคราะห์ด้วย SDS-PAGE เอนไซม์ที่ถูกทำให้บริสุทธิ์บางส่วนแสดงกิจกรรมสูงสุดที่อุณหภูมิ 70 องศาเซลเซียส และกิจกรรมของเอนไซม์ลดลงเมื่อพีเอชเพิ่มขึ้นจาก 5.5 ถึง 9.0 กิจกรรมของเอนไซม์เร่งได้โดยแคลเซียมคลอไรด์และมีกิจกรรมสูงสุดที่แคลเซียมคลอไรด์ 30 มิลลิโมลาร์ เอนไซม์ที่ถูกทำให้บริสุทธิ์บางส่วนสามารถย่อยแคปป้า-เคซีนได้อย่างรวดเร็ว และก่อให้เกิดเพปไทด์ที่มีขนาด 16 กิโลดาลตันและเอนไซม์ที่ผ่านการทำให้บริสุทธิ์บางส่วนจาก *Virgibacillus* sp. SK1-3-7 แสดงกิจกรรมการย่อย

สลายเคซีนต่ำ รูปแบบการย่อยสลายนมผงพร่องมันเนย และนมวัวพลาสเจอร์ไรซ์ โดยเอนไซม์จาก *Virgibacillus* sp. SK1-3-7 คล้ายกับเอนไซม์เรนนินทางการค้า

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2554

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SASITORN KAEWPHUAK : CHARACTERIZATION OF MILK-CLOTTING ENZYMES FROM BACTERIA ISOLATED FROM FISH SAUCE FERMENTATION. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 114 PP.

MODERATELY HALOPHILIC BACTERIA/*VIRGIBACILLUS*/MILK-CLOTTING ENZYME

Moderately halophilic bacteria isolated from fish sauce fermentation secreted extracellular proteinases exhibiting milk-clotting activities (MCA). The objectives of this study were to screen and identify bacteria isolated from fish sauce fermentation that showed the highest MCA and to characterize the milk-clotting enzyme. Among 19 isolates tested, isolates SK1-3-7, SK1-1-8 and SK39 exhibited the highest ratio of MCA to proteinase activity. Bacterial identification was performed using morphological and physiological characteristics. These 3 isolates were Gram-positive rods, endospore-forming and moderately halophilic bacteria. All isolates grew at 0-20% NaCl with optimum NaCl concentration of 5-15%. Isolate SK39 and SK1-1-8 grew at pH 6-10, while SK1-3-7 grew at pH 6-11. These 3 isolates grew at 10-45°C. The main quinone was menaquinone with seven isoprene units (MK-7) and the major cellular fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. Based on 16S rRNA gene sequences, the selected strains were a member of genus *Virgibacillus*. Strains SK 1-3-7, SK1-1-8 and SK39 showed 99.4, 99.6 and 99.6% similarity to *V. halodenitrificans*, respectively. The DNA-DNA relatedness between these strains and *V. halodenitrificans* JCM 12304^T obtained from DNA-DNA hybridization was 38.96-45.85%, suggesting

that SK1-3-7, SK1-1-8 and SK39 were a novel species of *Virgibacillus.*

Milk-clotting enzymes from *Virgibacillus* sp. SK1-3-7 were partially-purified and showed molecular mass of 20 and 36 kDa based on SDS-PAGE. The partially-purified enzymes showed optimum temperature at 70°C and activity decreased when pH increased from pH 5.5 to 9.0. These enzymes were activated by $CaCl_2$ showing maximal activity at 30 mM. The partially-purified enzymes of *Virgibacillus* sp. SK1-3-7 rapidly hydrolyzed κ-casein, producing a peptide with molecular mass of 16 kDa. The partially-purified enzymes showed low proteolytic activity towards casein. Hydrolytic pattern of skim milk and pasteurized whole milk by *Virgibacillus* sp. SK1-3-7 enzyme was similar to the commercial rennet.

School of Food Technology

Academic Year 2011

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

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Finally, I would like to dedicate this thesis to my family for their moral support and prayers. They have been a constant source of love, always believed in me and given me the great opportunity. I love them with all my heart.

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LIST OF ABBREVIATIONS

BLAST	=	Basic local alignment search tool
bp	=	Base pair
°C	=	Degree Celsius
CFU	=	Colony forming unit
dATP	=	Deoxyadenosine triphosphate
dCTP	=	Deoxycytidine triphosphate
dGTP	=	Deoxyguanosine triphosphate
dNTPs	=	Deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DNA	=	Deoxyribonucleic acid
EDTA	=	Disodiumethylenediaminetetraacetate
(m, μ) g	=	(milli, micro) Gram
h	=	Hour
(m, μ) l	=	(milli, micro) Liter
(m, μ) M	=	(milli, micro) Molarity
min	=	Minute
(m, μ) mol	=	(milli, micro) Mole
N	=	Normality
%	=	Percentage
PCR	=	Polymerase chain reaction
sp.	=	Species

CHAPTER I

INTRODUCTION

1.1 Introduction

Moderately halophilic aerobic endospore-forming, Gram-positive rod-shaped bacteria, are widely found in various saline environments, such as marine environments, salt lakes and salt fermented food. They are assigned to genera *Bacillus*, *Halobacillus*, *Virgibacillus*, *Filobacillus*, *Oceanobacillus*, *Lentibacillus*, *Paraliobacillus*, *Pontibacillus*, *Tenuibacillus*, *Salinibacillus*, *Alkalibacillus*, *Thalassobacillus* and *Piscibacillus* (Tanasupawat, Namwong, Kudo and Itoh 2007; Pakdeeto et al., 2007). Recently, *Piscibacillus* has been reported as the novel genera of moderately halophilic bacteria isolated from Thai fermented fish known as pla-ra (Tanasupawat, et al. 2007). *Virgibacillus siammensis* and *V. alimentarius* were proposed to be the new species of *Virgibacillus*, which were isolated from Thai fermented fish and Korean salt-fermented seafood (Tanasupawat, Chamroensaksri, Kudo and Itoh, 2010; Kim et al., 2011), respevtively.

Heyndrickx et al. (1998) firstly proposed the new genus of *Virgibacillus* from *Bacillus pantothenticus* based on data from the amplified rRNA gene restriction analysis, fatty acid profiles, SDS-PAGE patterns of whole cell proteins and phenotypic characterization. Description of *Virgibacillus* was later amended by Herman et al. (2003). Members of the genus are motile, Gram-positive or Gram- variable rods, endospore-forming, catalase-positive and they have a cell wall containing

peptidoglycan of meso-diaminopimelic acid type and DNA G+C contents ranging from 30.7 to 42.8 mol% (Wang, Chang, Ng and Chen 2008; Chen et al., 2008). Thus far, *Virgibacillus* comprises of 26 species. Numerous isolates of *Virgibacillus* obtained from fish sauce fermentation have not been fully characterized and identified. Phenotypic characterization, chemotaxonomy, 16s rRNA gene sequence and genomic DNA-DNA relatedness are important tools for bacterial identification.

Chymosin (E.C.3.4.23.4) is a milk-clotting enzyme derived from the fourth stomach of calf, which specifically hydrolyzes the peptide bond at Phe_{105}-Met_{106} of κ-casein, producing para-κ-casein and macropeptide (Fox and McSweeney, 1998). The hydrophilic macropeptides diffuse into the surrounding aqueous because of a reduction of steric stabilizing layer, while the para- κ-casein remains in the micelle core, resulting in aggregation of casein. Due to the limited supply of rennin, the cost of enzyme for cheese production is high. Animal, plant and microbial proteinases have been identified as a possible rennet replacement. Fungal proteinases from *Rhizomucor miehei*, *R. pusillus* and *Cryphonectria parasitica* have been commercially available and utilized as a chymosin substitute (Jacob, Jaros and Rohm, 2010). Most studies have focused on fungal milk-clotting enzymes because they show high milk-clotting activity and low proteinases activity. In addition to fungal milk-clotting enzymes, proteinases from bacteria have gained interest. Some bacterial proteinases, namely *Bacillus subtilis*, *B. mesentericus* and *B. cereus* have been reported as an alternative source of milk-clotting enzyme (Dutt, Meghwanshi, Gupta and Sexena, 2008). Ageitos, Vallejo, Sestelo, Poza and Villa (2007) reported that milk curd induced by *B. licheniformis* strain USC13 enzyme was white, firm, odorless and milk curd remained stable after 72 h. Milk-clotting enzyme from *B. subtilis* YB-3 was purified and

characterized. The enzyme exhibited high specificity to β-casein and yak milk (Li et al., 2012).

Proteinases from genus *Virgibacillus* have been reported in terms of production and characteristics including, *V. marismortui* NB2-1 isolated from Pla-ra (Chamroensaksri, Akaracharanya, Visessanguan and Tanasupawat, 2008), *V. panthenticus* isolated from fresh chicken meat (Gupta, Joseph, Mani and Thomas, 2008), *Virgibacillus* sp. isolated from Howz Soltan playa (hypersaline lake) (Rohban, Amoozegar and Ventosa, 2009) and *Virgibacillus* sp. SK33 and SK37 isolated from one month-old Thai fish sauce (Sinsuwan, Rodtong and Yongsawatdigul, 2007, 2008, 2010). *Virgibacillus* sp. SK33 and SK37 are a potential source of extracellular proteinases, which showed stability at high salt and were activated by NaCl.

Besides proteolytic activity, *Virgibacillus* sp. SK37 has been found to precipitate milk in skim milk agar. Several strains of *Virgibacillus* have been isolated from fish sauce fermentation at various time, and some strains showed ability to produce extracellular proteinase. It was postulated that some isolates could produce milk-clotting enzymes as an alternative source to chymosin. This would increase utilization of *Virgibacillus* proteinase. Thus far, the milk-clotting enzyme has never been studied in *Virgibacillus*. The milk-clotting enzyme produced from *Virgibacillus* can be served as an alternative for rennet. To successfully utilize *Virgibacilus* strain as a new source of milk-clotting enzyme, screening of proteinase with high milk-clotting activity and low proteolytic activity is needed. In addition, characteristics of the enzyme must be determined.

1.2 Research objectives

The objectives of this research were:

1) To screen milk-clotting enzyme from bacteria isolated from fish sauce fermentation,
2) To identify moderately halophilic bacteria producing milk-clotting enzyme, which were previously isolated from fish sauce using phonotypic, chemotaxonomic characteristics, DNA-DNA hybridization and 16S rRNA gene sequence,
3) To characterize milk-clotting enzyme from selected isolate.

1.3 Research hypotheses

In addition to fish protein hydrolysis, bacteria isolated from fish sauce fermentation secreted milk-clotting enzyme that showed similar caseinolytic activity to the commercial rennet. *Virgibacillus* isolated from fish sauce fermentation could be novel species.

1.4 Scope of the study

Bacteria isolated from fish sauce, which were able to produce milk-clotting enzyme were screened. Isolates showing the highest ratio of milk-clotting activity to proteolytic activity was selected as the potential strain. Identification and chacterization of the potential strain were carried out based on phenotypic and genotypic characterisitcs. Genotypic analyses included gene sequence analysis of 16S rRNA gene and DNA-DNA hybridization. In addition, fatty acid composition and G+C mol% were determined. Properties of milk-clotting enzyme, including effect of

pH and temperature on activity and hydrolysis pattern of milk protein, were also studied.

CHAPTER II

LITERATURE REVIEW

2.1 Casein micelles

Caseins are phosphoprotein containing approximately 80% of the total protein content of milk proteins (Brunner, 1997). They are precipitated from raw milk upon acidification at pH 4.6. Main components of casein are α_{s1}-, α_{s2}-, ß-, κ- and γ-casein and α-casein is a major casein (Walstra, Geurts, Noomen, Jellema and van Boekel, 1999). α-Casein contains two proteins, including α_{s1}- and αs_2-casein. α_{s1}-Casein has the highest charge and phosphate content, while α_{s2}-casein has the highest hydrophilic due to the presence of phosphoserine. ß-Casein is also phosphoprotein with a hydrophilic head and hydrophobic tail. Thus, α_{s1}-, α_{s2}- and ß-casein are sensitive to precipitation by calcium salts. κ-Casein is a glycoprotein containing carbohydrate groups, which are esterified to one of the threonines and has galactosamine, galactose, and one or two N-acetylneuraminic acid residues (Walstra et al., 1999). κ-Casein remains soluble in the presence of calcium salts and it plays an important role in stabilizing casein micelles (Walstra et al., 1999; Horne and Banks, 2004).

About 95% of casein in normal milk is not present in solution but in large colloidal particles known as micelles. Micelles contain on dry matter basis about 94% protein and 6% low molecular weight species as colloidal calcium phosphate (CCP), consisting of calcium, magnesium, phosphate and citrate (Fox and McSweeney, 1998). Casein micelles are generally spherical shape as observed by electron

microscopy with diameters ranging from 50-500 nm and masses ranging from 10^6-10^8 Da. They are able to scatter light resulting white color in milk (Fox and McSweeney, 1998). α, β and κ-Casein monomers form small and roughly spherical aggregates known as submicelles which are stabilized by hydrophobic interactions and calcium bridges. There are about 12-15 nm in diameter, each of them contains 20-25 casein molecules (Walstra et al., 1999). These submicelles contain a hydrophobic core and are covered by a hydrophilic layer. Submicelles are aggregated by colloidal calcium phosphate (CCP) linkages to form casein micelles. The κ-casein is located near the outside of the micelles with the hydrophilic part protruding from the micelles surface to form a hairy layer that will prevent aggregation of casein micelles by steric and electrostatic repulsion, resulting in stable casein micelles.

2.2 Mechanism of milk coagulation

Milk coagulation is the first step in cheese manufacture. Coagulation is essentially the formation of a gel by destabilizing the casein micelles causing them to aggregate and form a network which entraps fat. Coagulation can be initiated by:

A. Acidification

At natural milk pH of 6.7, caseins have a net negative charge causing electrostatic repulsion which stabilizes casein micelles. Acidification causes the casein micelles to destabilize or aggregate by decreasing their electric charge to that of the isoelectric point. As the charges of casein molecules are neutralized and electrostatic repulsion is reduced, hydrophobic interactions between caseins occurs resulting in aggregation of casein micelles to acid milk gel. This process is performedby acidogen performedby acidogen (usually gluconic acid-δ-lactone) or lactic acid bacteria which

produce lactic acid from lactose (Guinee and Wilkinson 1996; Lucey, 2004).

B. Enzyme coagulants

Enzyme induced gel formation of milk is initiated by proteolysis of κ-casein molecules, followed by calcium-induced micelles aggregation. Enzymes for milk coagulation come from a number of sources: animal, plants and microorganisms. Rennet from calf stomach is commonly used. This enzyme hydrolyzes peptide bond specifically the Phe_{105}-Met_{106} of κ-casein, producing para- κ-casein and macropeptide The hydrophilic macropeptides of κ-casein diffuse into the surrounding aqueous, which removes the steric stabilizing layer while the para-κ-casein remains to the micelles core. As a result, both negatively charged groups and steric stabilization reduces, resulting to aggregation into gel of para- κ-casein and other caseins under influence of Ca^{2+} (Walstra, 1999; Horne and Banks, 2004).

2.3 Milk-clotting enzymes

Milk-clotting enzymes are proteolytic enzyme, which are essential for cheese production and one of the most important enzymes in the food industry. Proteolytic enzyme can be classified, based on their catalytic activity to four groups: serine, cysteine, metallo and aspartic proteinases (Wong, 1995). Rennet is a milk-clotting enzyme widely used in cheese making, which is extracted from calf abomasums. The enzyme basically consists of chymosin and pepsin. Chymosin (EC 3.4.23.4) is aspartic proteinase with two essential aspertyl residues in active site. Chymosin has a molecular mass about 35600 Da and has an isoelectric point at about 4.65. Chymosin is the most stable at pH between 5.3 and 6.3, and lost activity at lower pH values (pH 3-4), probably due to auto-degradation. At higher pH values (above pH 9.8), activity

loss is due to an irreversible conformational change. When the temperature increases to 45-50˚C, activity of chymosin rapidly decreases (Crabbe, 2004; Walstra et al., 1999). This enzyme is active with high specificity for cleaving at Phe_{105}-Met_{106} peptide bond of κ-casein, resulting in destability of the casein micelles and aggregation into milk gel. In the 1960s, world cheese production had increased but rennet supply decreased because of the limited availability of calf stomachs (Horne and Banks, 2004). Alternative enzymes should have specific properties with high ratio of clotting activity (i.e. the specificity on κ-casein) to proteolytic activity. The most important rennet replacement derives from fungi including *Rhizomucor miehei*, *R. pusillus* and *Cryphonectria parasitica*. Recombinant proteinases by genetically modified microorganisms of *Aspergills, Kluyveromyces* or *Escherichia coli* have also been used commercially. In addition, plant proteinases from *Cynara cardunculus* has been reported to have milk-clotting ability (Jacob, Jaros amd Rohm, 2010; Merheb-Dini, Garcia, Penna, Gomes and da Silva, 2012).

Several microbial proteinases exhibit activity similar to chymosin and are suitable for cheesemaking. These include *Rhizomucor miehei*, *R. pusillus* and *Cryphonectria parasitica*, which produce aspartic proteinase by fermentation. These enzymes specifically cleave the Phe_{105}–Met_{106} bond of bovine κ-casein (Horne and Banks, 2004). A proteinase from *Cryphonectria parasitica* cleaves the Ser_{104}–Phe_{105} bond (Horne and Banks, 2004). The aspartic proteinase produced by *Rhizomucor miehei* has optimum milk clotting activity at pH 5.6 with a molecular weigh of 40.5 kDa (Preetha and Boopathy, 1997). The enzyme is the most common microbial coagulant for cheese making (Jacob et al., 2010). Recently, a proteinase from *R. pusillus* was reported to have molecular mass of 49 kDa with optimum activity at

50 ˚C and pH of 5.0 and $CaCl_2$ at 20 mM (Nouani et al, 2009). Recently, alternative sources of milk-clotting enzyme have been sought as shown in Table 2.1. In addition to fungal milk-clotting enzymes, proteinases from bacteria have gained interest. Several reports of milk-clotting enzyme from bacteria are limited to the genus *Bacillus*. Stoeva and Mesrob (1977) reported milk-clotting enzyme from *Bacillus mesentericus* 76, which was a typical alkaline proteinase with pH optimum of 8.5-9.0 and showed higher hydrolysis of casein than hemoglobin. Milk-clotting enzyme from *B. subtilis* K-26 was purified and showed a molecular mass of 27,000 Da. The enzyme was most stable at pH 7.5 and showed increasing clotting activity with a decrease in milk pH up to 5.0. The maximum milk-clotting activity was obtained at 60°C, but the enzyme was inactivated by heating at 60°C for 30 min (Rao and Mathur, 1979). Ageitos, Vallejo, Sestelo, Poza, Villa (2007) reported that milk curd produced from proteinase of *Bacillus licheniformis* strain USC13 are white, firm, odorless and remain stable after 72 h. Milk-clotting enzyme from *B. subtilis* YB-3 was purified and characterized. The enzyme showed molecular mass of 42 kDa, optimal activity up to 70°C with pH stability ranging from 5.0–9.0. The enzyme exhibited high specificity to β-casein and yak milk casein (Li et al., 2012).

Table 2.1 Microorganisms reported to produce milk-clotting enzymes.

Microorganism	Optimum condition			Mass	Distinct feature	Reference
	pH	Temp (°C)	$CaCl_2$ (mM)	kDa		
Myxococcus xanthaus	6	37	NA	40	Acceptable yield and properties of curd in cheesemaking experiment.	Poza et al. (2003)
Enterococcus faecalis	5.8	70	50	34-36	Similar electrophoretic patterns of hydrolysed κ-casein as *Rhizomucor miehei*, effectively applied in Camembert cheese manufacture.	Sato et al. (2004)
Bacillus licheniformis	6-7.5	30,37	NA	34,62	Show typical milk clotting kinetics, capable to produce milk-curd.	Ageitos et al. (2007)
B. sphaericus	6-7.5	55	40	55,47	-	EL-Bendary et al. (2007)
B. subtilis	6	70	NA	42	Show a potential to be used in yak cheese industry.	Li et al. (2012)
Thermomucor indicae-seudaticae N31	5.7	70	40	NA	Making good quality of Prato cheese, enzymatic hydrolysis of Proto cheese ripening did not differ from the commercial coagulant.	Merheb-Dini et al. (2010); Merheb-Dini, Garcia et al. (2012)
B. amyloliquefaciens D4	5.5	65	25	58.2	High level of milk-clotting activity and low level of thermal stability.	He et al., (2011);

NA, Not avilable

2.4 Halophilic bacteria

Halophilic bacteria are a group of microorganisms that inhabit and adapt to environments with high salinities (Ventosa, Márquez, Garabito and Arahal, 1998). Halophiles require salt for growth and the optimum salt concentration for growth often depends on the medium composition and growth temperature. Halophilic microorganisms have two strategies including accumulation of inorganic ions and accumulation of organic solute to balance their cytoplasm with the osmotic pressure (Oren, 2008). They are found in all three domains: Archaea, Bacteria and Eukaryote. They can be classified into 3 groups based on salt requirement for growth. These are: slight halophiles that grow optimally at 0.2–0.85 M (2–5% NaCl); moderate halophiles that grow optimally at 0.5-2.5 M (3-15% NaCl); extreme halophiles that live in natural environment of high salt concentration (5 M or 25% NaCl) and grow optimally at 3.4-5.1 M (20-30% NaCl). Halotolerant bacteria are able to grow both in the absence and in high salinity salts (or extremely haloterant if growth range extends above 2.5 M or approximately 15%) (Oren, 2008).

Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance for salt. Thus, they are widely distributed in hypersaline environments including natural hypersaline brines, in arid, saltern ponds, coastal and even deep-sea locations, as well as in artificial salterns used to mine salt from the sea (Turper and Galinski, 1986). Moreover, they can be found in many kinds of fermented foods, especially fermented fish and fish products, such as fish sauce, fermented fish pastes and salted fish (Ventosa, Nieto and Oren, 1998; Vihelmsson, Hafsteinwsson and Kristjansson 1996; Hiraga et al., 2005; Tanasupawat, Namwong, Kudo and Itoh, 2007; Nawong, 2006).

2.4.1 Halophilic bacteria in high salt environment

Halophilic bacteria maintain an osmotic balance of their cytoplasm with the hypersaline environment by two fundamentally different strategies to survive in saline environments (Oren, 2008). The "high-salt-in strategy" and "compatible-solute-in strategy" are used by halophilic microorganisms to balance their cytoplasm osmotically with their medium (Oren, 2008). Cell maintenance of high intracellular KCl concentration in cytoplasm for osmotic balance is called "high-salt-in strategy". The extremely halophilic archaea of family *Halobacteriaceae*, such as *Halobacterium salinarium* and *Haloarcula marismortui* is well understood in the maintenance of high intracellular KCl in cell. *Halobacterium* strain NRC-1 has shown the multiple transport systems, which are responsible for permeability of K^+ and Na^+. The transports include ATP-driven transport system, a low-affinity transporter driven by the membrane potential, TrkAH, an active Na^+ efflux (Ng et al., 2000). The extremely halophilic *Salinibacter rubber* accumulates very high concentrations of Cl^- and K^+/Na^+. The *S. ruber* genome analysis shows that potassium could be taken up via a TrkHA transport mechanism (Mongodin et al., 2005). The TrkA is the cytoplasmic membrane surface protein that binds NADH/NAD^+ and is essential for the transport activity (Saum and Müller, 2008). This strategy requires adaptation of the intracellular enzyme, as protein should maintain the activity of the cellular metabolic systems under high concentration of salts in the cytoplasm (Oren, 2008; Detkova and Boltyanskys, 2007). Most proteins of *Halobacteriales* contain a large excess of the acidic amino acids like glutamate and aspartate and a low content of the basic amino acids like lysine and arginine, which lead to a reduction of hydrophobicity of cytoplasmic proteins. This adaptation reduces the salting-out effects of K^+ and allows

the protein to retain its flexibility under extreme salinity (Martin, Ciulla and Robert, 1999). Thus, most proteins of bacteria having “high-salt-in strategy” denature when suspended in low salt environments.

The “compatible-solute-in strategy” is to exclude salt from cytoplasm and to biosythesize and/or accumulate of organic osmotic solute. Compatible solutes are polar, highly soluble molecules and do not interact with proteins; most of them are uncharged or zwitterionic including amino acids or amino acid derivatives, such as glutamine, glutamate, proline, ecotine, choline, betaine, and glycine betaine, as well as sugar and sugar alcohols, such as sucrose, trehalose, glycerol, arabitol, mannitol and erythritol (Oren, 2008; Galinski, 1995; Robert, 2005). This strategy has extensively been found in halophilic prokaryotes and eukaryotes. Table 2.2 shows compatible solutes that have been reported from prokaryotes. Glycine betaine is a common compatible solute found in plants, mammals, archaea and bacteria (Roeûler and Müller, 2001). Halophilics archaea can accumulate or *de novo* synthesize of glycine betaine. *Methanohalophilus portucalensis* synthesizes glycine betaine by reductive methylation of glycine and oxidation of exogenous choline, while halotolerant *Methanosarcina mazei* accumulate the compound from the medium when exposed to salt stress (Roeûler and Müller, 2001; Oren 2008). Halophilic bacteria *Salinivibrio costicola* has a membrane-bound choline dehydrogenase and a soluble betaine aldehyde dehydrogenase for conversion of choline to glycine betaine (Choquet, Ahonkhai, Klein and Kushner, 1991). The most widely found compatible solute in bacteria is ectoine. Various gram-negative and gram-positive bacteria produce ectoine via evolutionarily highly conserved *ectABC* gene cluster. Diaminobutyric acid transaminase (EctB), the diaminobutyric acid acetyltransferase (EctA), and the ectoine

synthase (EctC) are enzymes which convert aspartate semiadehyde to ectoine. Compatible solute as proline is found especially in the *Firmicutes* (Oren, 2008). Proline synthesis via three enzymes: γ-glutamyl kinase, γ-glutamyl phosphate reductase and Δ^1-pyrroline-5-carboxylate reductase, for conversion of glutamate to proline, which encode by an operon *proHJA.* In Gram-negative bacteria, proline was taken from the medium by three transporters (PutP, ProP and ProU) (Sleator and Hill, 2001). The biosynthesis of glutamate is regulated by gene *gltAB1B2* and a*dh1h2* that encode glutamate synthase and glutamate dehydrogenase, while glutamine is synthesized by glutamine synthase from gene g*lnA1A2*. The production of glutamate depends on chloride ion. The accumulation of intracellular Cl^- will activate the transcription of glnA2 and later increase glutamine synthase activity, and increase glutamate and glutamine (Saum and Müller, 2007). The intracellular concentration of solute can result osmotic equilibrium with the external salt concentration without destructive effect to intracellular proteins, thus microorganisms can adapt to surprisingly broad salt concentration range. (Oren, 2008; Ventosa et al., 1998).

Table 2.2 Compatible solutes detected in prokaryotes.

Compatible solute	Genus and species
Ectoine	*Halomonas elongata, Ectothiorhodospira halochloris, H. boliviensis, Brevibacterium epidermis, Chromohalobacter israelensis, C. salexigens*
Hydroxyectoine	*H. elongata, Nocardiopsis halophila,* aerobic heterotrophic bacteria
Betaine	*Actinopolyspora sp., Halorhodopira halochloris, Thioalkalivibrio versutus*
α –Glutamate	Marine bacteria, some methanogenic archaea

Table 2.2 (Continued)

Compatible solute	Genus and species
Proline	*Streptomyces,Salinicoccus roseus, Salinicoccus hispanicus,* halophilic/halotolerant Bacillus strains
Glycine betaine	Photosynthetic purple bacteria and halophilic cyanobacteria
Mannosylglycerate	*Methanothermus fervidus, Pyrococcus furiosus, Rhodothermus marinus,Thermus thermophilus, Pyrococcus furiosus,Thermococcus spp.*
Diglycerol phosphate	*Archaeoglobus fulgidus*
Mannosylglyceramide	*Rhodothermus marinus*
Glucosylglycerate	*Agmenellum quadruplicatum, Erwinia chrysanthemi, Stenotrophomonas maltophilia*
Trehalose	*Pyrobaculum aerophilum, Thermoplasma acidophilum, Actimopolyspora halophila, Rubrobacter xylanophilus*
Sucrose	*Anabaena (blue green algae), Nitrosomonas europaea and proteobacteria*
Mannitol	*Pseudomonas putida*

Modified from: Shivanand and Mugeraya (2011)

2.5 Fish sauce

Fish sauce is the most important fermented fish products used as a condiment in Southeast Asia. Fish sauce is called by different names depending on the country of origin. In Malaysia, fish sauce is called budu; in the Philippines, patis; in Indonesia, Ketjap-ikan; in Burma, ngapi; in Cambodia and Vietnam, nouc-mam (or nouc-nam); in Thailand, nampla; in Japan, ishiru or shottsuru; in India and Pakistan, colombo-cure; in China, yeesu; and in Korea, aekjeot (Lopetcharat, Choi, Park, and Daeschel, 2001). These sauces are a clear brown liquid and mild cheesy/salty flavor used as a

flavor enhancer or salt replacement in various food preparations and it contains a mixture of all essential amino acids and other protein degradation produced by hydrolysis of fish. (Lopetcharat et al., 2001; Taira, Funatsu, Satomi, Takano, and Abe, 2007; Tungkawachara, Park, and Choi, 2003)

Fish and salt are 2 major ingredients in fish sauce production. Thai fish sauce is mainly produced from anchovies (*Stolephorus* spp.), mackerel (*Ristrelliger* spp.), and herring (*Clupea* spp.) (Lopetcharat and Park, 2002). Generally, nampla is produced by mixing fish and salt in the ratio of 2:1 or 3:1 (fish:salt) (w/w), depending on area of production and fermenting at ambient temperature (30-40°C) for 12-18 months in an underground concrete tank (Lopetcharat and Park, 2002; Tungkawachara et al., 2003). During fermentation, fish proteins are hydrolyzed by endogenous fish and microbial proteinases. The liquid from the fermentation tank is filtered to remove residual solid fraction and transferred to the ripening tank. After 2-12 weeks of ripening, the first grade nampla is obtained (Wilaipan, 1990; Lopetcharat et al, 2001). Typically, chemical composition of the first grade fish sauce contains about 20 g/L of total nitrogen (16 g/L from amino acids), 25-28% of salt, 0.2-0.7% of ammonium, and pH 5.1-5.7 (Park et al., 2001).

2.5.1 Halophilic bacteria in fish sauce

The high concentration of salt (25-30%) in fish sauce limits groups of microorganism that can thrive during fermentation. Most microorganisms found in fish sauce are classified as halotolerant, slightly halophilic, moderately halophilic, and extremely halophilic bacteria. These are microflora from fish, solar salt, and fermentation tank (Lopetcharat et al., 2001). Bacteria found in fish sauce fermentation play an important role in flavor/aroma development and involvement of protein

hydrolysis during fermentation. Halophilic bacteria found in fish sauce fermentation include moderately halophilic bacteria; *Lentibacillus* (Tanasupawat, et al., 2006), *Filobacillus* (Hiraga et al., 2005), *Halobacillus* sp. SR5-3 (Namwong et al., 2006), *Halobacillus* (Chaiyanan, Maugel, Hug, Robb, and Colwall, 1999), *Tetragenococcus* (Thongsanit, Tanasupawat, Keeratipibul, and Jatikavanich, 2002), *Virgibacillus* (Nawong, 2006), *Chromohalobacter* (Tanasupawat, Namwong, Kudo, and Itoh, 2009), and extremely halophilic bacteria; *Halobacterium* (Thongthai, McGenity, Suntinanalert, and Grant, 1992; Tanasupawat et al., 2009), *Halococcus* (Namwong, Tanasupawat, Visessanguan, Kudo, and Itoh, 2007) and *Natrinema* (Tapinkae et al., 2008).

Bacterial enzymes play an important role during fish sauce fermentation. Some amino acids can be used as substrates for lactic acid bacteria. Norberg and Hofsten (1968) found *Halobacterium* sp. and *Halococcus* sp. during fish sauce fermentation and are classified as extremely halophilic bacteria that showed ability in gelatin and casein hydrolysis. *Tetragenococcus halophilus* and *T. muriaticus* hydrolyzed casein (Tanasupawat et al., 2003; Thongsanit et al., 2002; Kobayashi et al., 2003). *Halobacterium salinarium* and *Hbt. cutirubrum* produced extracellular proteinase at 25% NaCl (Ihara, Wanatabe, and Tamura, 1997; Thongthai et al., 1992; Thongthai and Suntinanalert, 1991). *Halobacillus thailandensis* sp. nov. isolated from fish sauce and secreted metalloproteinase (Chaiyanan et al.,1999). Hiraga et al. (2005) and Namwong et al. (2006) purified and characterized a serine proteinase from *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3, respectively, which activity increased with NaCl concentration. *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 isolated from 1-month-old fish sauce fermentation produced NaCl-stable serine

proteinases and NaCl-activated serine proteinases, respectively (Sinsuwan, Rodtong and Yongsawatdigul, 2007, 2008). Moreover, these strains (SK37 and SK33) showed high proteolytic activity towards anchovy substrate and could be promising strains for starter culture development for fish sauce fermentation.

2.6 Bacteria in the genus *Virgibacillus*

2.6.1 Characterization and identification

Heyndrickx et al. (1998) proposed to transfer *Bacillus pantothenticus* to the genus *Virgibacillus* as *Virgibacillus pantothenticus* on the basis of amplified DNA restriction analysis, fatty acid profiles, SDS-PAGE pattern of whole-cell protein and phenotypic characteristics. This genus could be distinguished from members of *Bacillus* rRNA and from members of *Paenibacillus* and other aerobic endospore-forming bacteria, such as *Halobacillus*, *Paenibacillus*, *Brevibacillus* and *Aneurinbacillus* by routine phenotypic tests. In addition, two species of *Salibacillus* were reclassified to *Virgibacillus* based on of genotypic and phenotypic characteristics (Heyrman et al., 2003). Moreover, Yoon, Oh and Park (2004) proposed to rename *Bacillus halodenitrificans* to *Virgibacillus halodenitrificans* based on 16S rRNA gene comparisons.

Virgibacillus are Gram positive rods, 0.3-0.8 μm×2-8 μm in size. Cell arrangement is single, pair, or short chains, or filaments. They bear spherical to ellipsoidal endospores that are located at terminal or subterminal position. Colony is circular and slightly irregular, smooth, glossy, low convex and slightly transparent to opaque. They are aerobic or weakly facultatively anaerobic, catalase positive and motile. Casein, gelatin and aesculin are hydrolyzed by most species, sometimes

weekly. Growth of *Virgibacillus* is simulated by 4-10% NaCl. Many species tolerate high salt concentration of 20-25% and do not grow in the absence of salt. They have DNA G+C content of 30.7 to 42.8 mol %, cell wall peptidoglycan contains meso-diaminopimelic acid and major fatty acids in cell membrane are anteiso-$C_{15:0}$. The main menaquinone type of *Virgibacillus* is MK-7 (94-99%), with minor to trace amounts of MK-6 and MK-8, which are similar profile to most other aerobic endospore-forming bacteria (Heyrman, Vos and Logan, 2003; Chen *et al*., 2008).

The genus *Virgibacillus* are moderately halophilic or halotolerant bacteria and currently consists of 26 recognized species, namely *V. pantothenticus* (Heyndrickx et al., 1998), *V. proomii* (Heyndrickx et al., 1999), *V. carmonensis*, *V. marismortui*, *V. necropolis* and *V. salexigens* (Heyrman et al., 2003), *V. halodenitrificans* (Yoon et al., 2004), *V. dokdonensis* (Yoon, Kang, Lee, Lee, and Oh, 2005), *V. koreensis* (Lee et al., 2006), *V. halophilus* (An, Asahara, GoTo, Kasai and Yokota, 2007), *V. olivae* (Quesada, Aguilera, Morillo, Ramos-Cormenzana and Monteoliva-Sa´nchez, 2007), *V. salarius* (Hua et al., 2008), *V. chiguensis* (Wang et al., 2008), *V. kekensis* (Chen et al., 2008), *V. salinus* (Carrasco, Márquez and Ventosa, 2009), *V. lioralis* (Chen, Liu et al., 2009), *V. sediminis* (Chen, Cui et al., 2009), *V. zhanjiabgensis* (Peng ea al., 2009), *V. arcticus* (Niederberger et al., 2009), *V. xinjiangensis* (Jeon et al., 2009), *V. siammensis* (Tanasupawat, Chamroensaksri, Kudo and Itoh, 2010), *V. byusanensis* (Yoon et al., 2010), *V. subterraneus* (Wang, Y Xue and Ma, 2010) *V. soli* (Kämpfer et al., 2011), *V. alimentarius* (Kim et al., 2011) and *V. campisali* (Lee, Kang, Oh and Yoon, 2011). Out of a total of 26 recognized species, 18 of which were isolated from saline environments, such as saline lakes, saltern ponds, desert, hypersaline soils, and which including salted food. Table 2.3 shows some characteristics of *Virgibacillus* spices

were isolated from saline environments.

Many bacteria can be identified based on phenotypic characteristics, which have been performed using traditional microbiological methods or commercial available kits. Results are compared with database of known bacteria (Brown and Leff, 1996). Heyndrickx et al. (1998) used API 20E and 50 CHB for testing acid producing ability of *V. pantothenticus*. Subsequently, Heyrman et al. (2003) added 7% NaCl in the API 20E and 50 CHB to elucidate phenotypic characteristics of halotolerant and halophilic *Virgibacillus*. But phenotypic characteristics alone still have limitation for species identification because other taxa of aerobic endospore-forming bacteria share many common phenotypic properties. In addition, *Virgibacillus* are wrongly identified because they are not included in the database of commercial test kits. Thus, other techniques including molecular genetics and chemotaxonomy should be applied.

Molecular genetic techniques used to distinguish bacterial species include 16S rRNA gene sequencing and DNA-DNA hybridization. The 16S rRNA gene sequencing is a powerful tool and is a common molecular technique used for bacterial species identification (Wilson, 1995: Priest, F and Austin, B. (1993).). The polymerase chain reaction (PCR) technique and a selection of universal bacteria primer are used widely for the study of rRNA genes amplified (McCabe, Zhang, Huang, Wager and McCabe, 1999). The amplified DNA can be sequenced directly or cloned into a plasmid vector. The sequence is compared with well known databases using analysis software.

DNA-DNA hybridization is the standard for describing new species (Stackebrandt et al. 2002). This technique is based on comparison between whole

genome of two bacteria. DNA probe binds to a complementary region of single-stranded DNA, depending on the similarity of their nucleotide sequences and the degree of relatedness, usually expressed as %similarity. A novel species of any bacteria after identified using 16S rRNA gene sequence should be confirmed by DNA/DNA hybridization. Quesada et al. (2007) isolated the strain E308^T from waste wash-water from processing of Spanish-style green olives. Based on 16S rRNA gene sequence, the isolate showed the highest level of similarity to *V. marismortui* (99%) and 96% similarity to *V. proomii*, *V. dokdonensis* and *V. pantothenticus*. When results were confirmed by DNA/DNA hybridization, the strain E308^T showed low level of similarity with the type strain of *V. marismortui* (46.5%), which was below 70 %. Thus, the strain E308^T represented a novel species of the genus *Virgibacillus*, and named as *V. olivae*. Based on this study, the combination between 16S rRNA gene sequence and DNA/DNA hybridization increased accuracy in the identification. Wang et al. (2008) identified *V. chiguensis* using 16S rRNA gene sequence technique and confirmed the species by DNA/DNA hybridization technique. Moreover, they used phenotypic characteristics of growth at high concentration of NaCl up to 30% as a criterion for species differentiation. Therefore, 16S rRNA gene sequence, DNA-DNA hybridization and phenotypic characterization are required for accurate bacterial identification.

Table 2.3 Characteristics of *Virgibacillus* species isolated from saline environments.

Characteristic	Type culture strain[d]																	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Habitat	marine solar saltern	Chigu saltern	salt lake	salt field	saline soil	Dead sea	salt lake	solar saltern	Thai fermented fish	salt lake	saline lake	subsurface saline soil	marine solar saltern	marine solar saltern	salt-fermented seafood	permafrost core (Canadian high Arctic)	salt lake	sea water
Spore shape[a]	E	S, E	E, S	E	E, S	E	E, S	E	E	E, S	E	E, S	S	S, O	E	E	E	E, S
Spore position[b]	S,T	T, S	T	T	T	S	S	C, S, T	S	T, S	S, T	T	T	T	T, S	C, S	S	T
Gram stain	v	+	+	+	+	+	+	+	+	+	+	+	v	v	+	+	+	+
Colony pigmentation	-	-	-	-	Cream	-	White	-	Red	Cream	Cream	-	-	-	Cream	Light pink	-	Cream
Anaerobic growth	+	+	-	+	ND	-	-	-	+	-	-	-	+	-	-	+	-	ND
Growth in the presence of:																		
0.5 % NaCl	v (-)	+	+	w	-	-	+	-	-	+	-	+	+	+	+	+	-	-
25 % NaCl	v(+)	+	+	-	+	-	+	w	-	-	-	+	-	-	+	-	-	-
pH 10	-	-	+	-	+		+	+	-	-	+	-	-		+	-	+	+
Growth temperature (°C)	10-45	15-55	10-50	10-45	10-45	15-50	10-15	15-45	15-40	8-52	10-40	10-50	15-40	4-45	4-40	0-30	10-55	10-45
Nitrate reduction	+	+	+	-	+	+	-	-	-	+	+	+	ND	ND	ND	+	+	+
H_2S production	-	-	-	-	-	+	-	+	+	ND	-	+	ND	ND	ND	-	-	-
Hydrolysis of:																		
Aesculin	-	+	-	+	-	+	+	+	-	-	+	+	-	-	ND	ND	+	+
Casein	+	+	-	+	-	+	+	+	w	+	-	-	-	-	-	+	-	-
Gelatin	+	+	-	-	+	+	w	+	+	-	-	-	-	-	-	+	+	+
Strach	-	+	+	ND	-	ND	-	ND	+	-	-	-	ND	+	-	ND	-	-
Growth on:																		
D-Glucose	+	+	+	ND	+	+	ND	w	ND	+	-	+	+	-	ND	+	+	+
D-Fructose	+	v (+)	-	ND	+	+	+	+	ND	+	-	+	-	ND	ND	ND	-	-
Sucrose	+	+	-	ND	ND	+	ND	+	ND	ND	+	+	-	-	ND	+	-	-

Table 3 (Continued)

Characteristic	Type culture strain[d]																	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Acid production from:																		
N-Acetylglucosamine	+	-	-	-	ND	+	+	w	ND	ND	ND	+	ND	ND	+	-	+	ND
D-Galactose	+	+	-	-	+	-	-	w	-	-	+	+	-	ND	-	ND	-	-
D-Glucose	+	+	+	w	+	+	+	w	+	+	+	+	-	-	-	w	+	+
D-Fructose	v	+	-	+	-	+	+	w	-	+	+	+	-	w	-	w	+	-
D-Mannose	+	+	-	-	+	+	+	w	-	-			-	-	-	w	+	-
D-Trehalose	+	-	w	w	-	-	-	-	-	ND	+	-	-	-	-	+	ND	-
D-Mannitol	+	w	w	-	+	-	-	-	ND	-	-	-	-	ND	ND	+	-	-
Major polar lipids[c]	PG, DPG	PG, DPG,PE, PLs	PG, DPG, PLs	PG, DPG, PLs	PG, DPG	PG, DPG, PE	DPG, PG, PE	PG, DPG	PG, DPG	PG, DPG	PG, DPG	PG, DPG	DPG, PG, PLs	ND	DPG, PG, PE	PG, DPG, PLs	DPG, PG, PE, PLs	PG, DPG
DNA G+C (mol %)	38-39	37.3	41.8	41	40.2	39.0-42.8	37.3	36.3-39.5	38	44.5	38.8	37.1	39.5	37.6	37	38.2	40.9	39.5

Note: ND, no data available; w, weakly positive; v, variable; [a]E, Ellipsoidal; S, spherical; O, oval; [b]C, Central; S, subterminal; T, terminal

[c]DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, Phosphatidylglycerol; PLs, unidentified phospholipids

[d]1. *V.halodenitrificans* (Yoon et al., 2004), 2. *V. chigensis* (Wang et al., 2008), 3. *V. kekensis* (Chen et al., 2008), 4. *V. koreensis* (Lee et al., 2006), 5. *V. lioralis* (Chen, Liu et al., 2009), 6. *V. marismortui* (Arahal et al., 1999), 7. *V. salaries* (Hua et al., 2008), 8. *V. salexigeus* (Garabito et al., 1997), 9. *V. siammensis* (Tanasupawat et al., 2010), 10. *V. xinjiangensis* (Jeon et al., 2009), 11. *V. salinus* (Carrasco et al., 2009), 12. *V.subterraneus* (Wang et al., 2010), 13. *V. campisalis* (Lee et al., 2011), 14. *V. byunsanensis* (Yoon et al., 2010), 15. *V. alimentarius* (Kim et al., 2011), 16. *V. arcticus* (Niederberger et al., 2009), 17. *V. sediminis* (Chen, Cui et al., 2009), 18. *V. zhanjiangensis* (Peng et al., 2009)

2.6.2 Proteinase from *Virgibacillus*

Virgibacillus showed ability to produce extracellular proteinase. Rohban, Amoozegar, and Ventosa (2009) isolated moderately and extremely halophilic bacteria from a hypersaline lake in Iran. Some isolates were identified as *Virgibacillus* and were able to produce a great variety of hydrolytic enzymes, such as amylase, proteinase, cellulase, and inulinase. Proteinases from *Virgibacillus* show different characteristics and are summarized in Table 4. Extracellular proteinase of *V. marismortui* NB2-1 isolated from Pla-ra showed strong extracellular proteolytic activity that was active in extreme conditions and could be a potential source of thermoactive alkaline proteinase production (Chamroensaksri, Akaracharanya, Visessanguan, and Tanasupawat, 2008). Gupta, Joseph, Mani, and Thomas (2008) reported a proteinase from *V. pantotheticus* MTCC 6729, which was isolated from chicken meat samples. The enzyme was alkaline serine proteinase, with thermo-stable and compatible with commercial detergents showing the potential as a detergent additive. Proteinases from *Virgibacillus* sp. SK33 and SK37 isolated from one-month-old Thai fish sauce have been reported (Sinsuwan, Rodtong, and Yongsawatdigul, 2007, 2008a, 2008b, 2010; Phrommao, Rodtong, and Yongsawatdigul, 2011). In SK33, extracellular enzyme showed high stability towards various organic solvents at concentrations up to 25% (v/v) (Sinsuwan, et al., 2010). Crude extracellular and cell-bound proteinases of SK37 were subtilisin-like serine proteinases and were NaCl-activated enzyme with stability up to 25% NaCl (Sinsuwan, et al., 2011). It should be noted that proteinases from various species of *Virgibacillus* showed different characteristics (Table 2.4).

Table 2.4 Biochemical characteristics of extracellular proteinases from various species of *Virgibacillus*.

Species	Classification	Optimum activity			Mass (kDa)	Reference
		Temp (°C)	pH	NaCl (%)		
V.pantothenticus MTCC 6729	Alkaline serine proteinase	50	10	-	-	Gupta et al. (2008)
V.marismortui NB2-1	Alkaline serine proteinase	50	10	5	17, 19, 24, 29, 35	Chamroensaksri et al. (2008)
Virgibacillus sp. SK 33	Subtilisin-like serine proteinase	55	7.5	20-25	19, 32	Sinsuwan et al. (2008)
Virgibacillus sp. SK 37	Subtilisin-like serine proteinase	55-60	8	20-30	19, 34, 44	Phrommao et al.(2011)
V.halodenitrificans TKNR 13-3	Chymotrypsin-like serine proteinase	60	8	15	12, 21, 29, 39, 49	Tanasupawat, et al. (2011)

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganisms and cultivation

Eight strains of *Virgibacillus* sp., one strain of *Brevibacterium* sp. and 10 strains of *Staphylococcus* sp., were all isolated from fish sauce fermentation and were used for the screening of milk-clotting enzyme producing ability. Purified bacteria were maintained on JCM 168 medium (0.5% casamino acids, 0.5% yeast extract, 0.1% sodium glutamate, 0.3% tri-sodium citrate, 0.2% KCl, 2% $MgSO_4 \cdot 7H_2O$, 0.036% $FeCl_2 \cdot 4H_2O$, 0.00036 $MnCl_2 \cdot 4H_2O$ and 5% NaCl) and kept at -20°C with the addition of skim milk to the final concentration of 5%. For cell propagation procedure, the stock cultures were taken from -20°C, thawed at room temperature. Two hundreds µl of each culture were inoculated into 2 ml of JCM 168 broth 5% NaCl. After incubation at 35°C for 2-3 days, the culture was streaked on JCM 168 agar 5% NaCl and incubated under aerobic condition at 35°C for 2-3 days. Single colony was collected for further study.

3.2 Screening of bacteria producing milk-clotting enzyme

Screening of milk-clotting enzyme was carried out using a plate diffusion assay on casein agar (Atlas and Parks, 1997). The purified isolates were inoculated to Y-broth (1% yeast extract, 0.3% trisodium critrate, 0.2% KCl, 2.5% $MgSO_4 \cdot 7H_2O$ and 5%

NaCl) and incubated under aerobic condition at 35°C for 3 days. Crude enzymes were collected by centrifugation at 10,000 ×g for 30 min at 4°C. Casein agar wells were prepared using a sterile no.6 cork borer. Thirty-five µl of crude enzyme was added into a well and were incubated at 37°C for 24 h. Diameters of casein precipitate and clear zone were measured. Strains showing casein precipitate zone were selected for milk-clotting activity measurement.

3.3 Determination of proteolytic activity

Proteolytic activity was determined using azocasein (Sigma Chemical Co.; St. Louis, Mo., USA) as a substrate according to An et al. (1994). Crude enzyme (200 µl) was added to 2 mg of azocasein and McIlvaine buffer (pH7.0) in a final volume of 1 ml. Reaction mixture was incubated at 37°C for 30 min and terminated by adding 500 µl cold 50% trichloroacetic acid (TCA). The reaction mixtures were then incubated at 4°C for 15 min. Supernatants were collected by centrifugation at 10,000 ×g for 10 min and were added 100 µl of 10 N NaOH before measuring the absorbance at 450 nm (Smartspec^{TM} Plus Bio-Rad Laboratory, Hercules, U.S.A.). Blanks were prepared by adding TCA before adding crude enzyme. One enzyme unit was defined as the difference between absorbance sample and blank of 0.01 in 1 min.

3.4 Determination of milk-clotting activity

Milk-clotting assay was carried out according to Arima et al. (1970) with slight modifications. Crude enzyme (0.1 ml) was added to 1 ml of 10% skim milk (MercK CoA, Darmstadt, Germany) suspension containing 0.01 M $CaCl_2$ and incubated at 37°C. Time of initial curd formation was recorded. One milk-clotting unit is defined

as the amount of enzyme that clots 1 ml of substrate within 40 min and was calculated according to Merheb-Dini et al. (2010):

$U = 2400/T \times S/E$,

Where T is the time of initial curd formation, S is milk volume in 1 ml and E is enzyme volume in 0.1 ml.

3.5 Identification of selected milk-clotting enzyme-producing bacteria

Isolates showing the highest milk-clotting activity were identified using phenotypic, biochemical, physiological, chemotaxonomic, ribosomal RNA gene and DNA-DNA hybridization.

3.5.1 Morphological characteristics

Cells were cultivated on JCM168 broth containing 5% NaCl and incubated at 37°C for 3 days. Gram stain, cell morphology and spore were examined by light microscopy (Cappuccino and Sherman, 1967).

3.5.2 Physiological characteristics

3.5.2.1 Catalase

Bacterial cells were transferred to the surface of a glass slide and 3% hydrogen peroxide was dropped onto glass slide. Positive result showed rapid gas formation for catalase test.

3.5.2.2 Oxidase

Oxidase test was determined by dropping 1% of tetramethyl-p-phenylenediamine dihydrochloride on filter paper (Whatman no.4) and the bacterial cells were streaked on the filter paper. Appearance of dark blue indicated a positive result.

3.5.2.3 Extracellular enzyme

Skim milk agar and starch agar with addition of 5% (w/v) NaCl were also used to evaluate the ability to hydrolyze casein and starch, respectively. Isolates were point inoculated on these media and incubated at 37°C for 2-3 days. The clear zone around colony indicated hydrolysis of casein. Starch hydrolysis was detected by adding iodine solution on plate and clear zone around colony indicated the positive result for starch hydrolysis.

3.5.2.4 Oxidation-fermentation

Oxidation fermentation medium (O/F medium) containing 5% NaCl was used for oxidative and fermentative metabolism of glucose. Isolates were stabbed into two tubes of O/F medium and steriled mineral oil (2-3 ml) was added to one of the tubes. The inoculated samples were incubated at 35°C for 1-2 days. Yellow medium indicated glucose fermentation.

3.5.2.5 Nitrate reduction

Nitrate reduction test was performed using the API 20E systems (bioMe´rieux, Lyon, France) according to the instruction manual.

3.5.2.6 Hydrogen sulfide production

Hydrogen sulfide production was performed using the API 20E systems (bioMe´rieux, Lyon, France) according to the instruction manual.

3.5.2.7 Carbohydrate fermentation

Carbohydrate fermentation tests were performed using the API 50 CHB systems (bioMe´rieux, Lyon, France) according to the instructions manual. All suspension media used in the experiment contained 5% NaCl (w/v).

3.5.2.8 Growth at different NaCl concentrations, pHs and temperatures

Bacterial growth was investigated in trypticase soy broth (TSB) at different NaCl concentrations, pHs and temperatures. Inoculum with approximate cell counts of 10^6 CFU/ml was transferred to TSB at each condition. Growth at each condition was repeated for 3 cycles. Positive results were assigned when growth in all 3 cycles was observed.

Bacterial growth at various NaCl concentrations (0, 0.5, 3, 5, 10, 15, 20, 25 and 30%) was observed in TSB at pH 7.0. Inoculum (500 µl) with approximate cell counts of 10^6 CFU/mL were transferred to 5.0 mL of TSB. Each condition was analyzed in duplicate and incubated at 35°C for 2-14 days. Optical density at the wavelength of 600 nm was used to evaluate bacterial all growth evaluation. Samples with positive result were transferred to 5 ml of fresh TSB and incubated at the same condition for 2 more cycles.

For growth at different pHs, TSB broth containing 5% NaCl at various pHs of 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 were prepared. Inoculum size of 1% were used and added to 5.0 mL of TSB for each test and incubated at 35°C. Bacterial evaluation was determined as described above.

For the effect of temperature, TSB containing 5% NaCl, pH 7.0 was incubated at 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50°C. Evaluation of bacterial growth was determined as described above.

3.5.3 Determination of ribosomal RNA gene sequences

3.5.3.1 DNA extraction

DNA extraction was carried out according to Saito and Miura (1963). DNA was extracted from cell grown on TSA with containing 5% NaCl at 37°C for 2

days. Bacterial cells at late exponential phase were harvested and dissolved in 1 ml saline-EDTA (pH 8.0). Cell suspentions were lyzed using 0.75 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO., USA) at 37°C for 1 h followed by incubation at 60°C for 5 min with 250 µl of Tris-HCl pH 9.0 and 125 µl of 10% SDS. Phenol:chloroform (1:1) was added at equal volume to sample for protein removal. The upper layer was collected after centrifugation at 10,000 rpm for 20 min. DNA was precipitated by adding cold absolute ethanol and further purified using RNase (Sigma-Aldrich, St. Louis, MO., USA) and 100 µl of proteinase K (MerkKGoA, Darmstadt, Germany) at 37°C for 1 h. After 1 h, the proteins were removed again with phenol:chloroform (1:1) and upper layer was collected. DNA was precipitated by cold absolute ethanol and stored at -20°C.

3.5.3.2 PCR amplification of 16s rRNA gene

Reaction mixture contained 5 µl of 10×PCR buffer (200 mM Tris-HCl, pH8.0, 500 mM KCl), 2 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTP (dATP, dCTP, dGTP, dTTP) (Invitrogen™ life Technologies, Foster, CA., U.S.A), 1 µl of 20 µM of each forward and reverse primer (fd1 and rp2, Sigma Proligo, Helios, Singapore) (Table 3.1), 0.3 µl of 50 U Taq polymerase (Invitrogen™ life Technologies, Foster, CA., U.S.A.) and 1 µl of 150-200 µg genomic DNA template. The mixture was carried out by a thermo cycler (Thermo electron corporation Px2 Thermo cycler, San Francisco, CA., U.S.A.) and programs were set as follows: initial denaturation step at 95°C for 2 min; 35 cycles of denaturation step at 95°C for 45 sec; annealing step at 72°C for 2 min; and final extention step at 72°C for 10 min. PCR products were separated using 1% agarose gel electrophoresis and detected by ethidium bromide.

3.5.3.3 Cloning of 16s rRNA gene

The 16s rRNA gene was loaded into 1.2% agarose gel electrophoresis and detected by ethidium bromide. The 16s rRNA gene was excised from agarose gel using a clean razor blade and transfered to a microcentrifuge tube. The 16s rRNA gene was purified using a Wizard Gel/PCR product kit (Promega Corporation, Madison, WI., U.S.A.) and ligated into pGEM-T easy vector (Promega corporation, Madison, WI., U.S.A.) The recombinant vector was transformed into *E. coli* DH5α. Blue-white colony of transformed *E.coli* DH5α was selected (Sambrook and Russell, 2001). Wizard DNA purified kit was also used for plasmid vector purification. The vector was cut using EcoRI and incubated at 37°C for 4 h. DNA fragments were detected on 2% agarose gel electrophoresis.

Table 3.1 Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA gene.

Primer	Primer sequence (5′ to 3′)	Target region	Reference
16S rRNA gene amplification			
fD1	5′AGAGTTTGATCCTGGCTCAG-3′	8-27	Weisburg et al. (1991)
rP2	5′-ACGGCTACCTTGTTACGACTT-3′	1490-1511	Weisburg et al. (1991)
Nucleotide sequencing			
T7	5′-TAATACGACTCACTATAGGG -3′	53-72	Hans et al. (2002)
SP6	5′-TAATACGAC TCACTATAGGG -3′	2896-2916	Hans et al. (2002)
Forward primer	5′-TAACTACGTGCCAGCAGCC-3′	515-533	

3.5.3.4 Nucleotide sequence analysis and phylogenetic tree construction

The pGEM plasmid was used as a template for sequencing of cloned 16S rRNA fragments with T7/SP6 primer (Table 3.1). Sequencing was done using ABI377 automated DNA sequencer (Perkin Elmer, Applied Biosystems, Inc., Foster, CA., USA). The sequences were compared to nucleotides database provided by the National Center for Biotechnology Information (NCBI) using the BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The alignment scores and the percent sequence identity were determined for the closest identity of the sequences obtained. The multiple sequence alignment was performed using CLUSTAL_X (Thompson, Gibson, Plewniak, Jeanmougin, and Higgins, 1997). Phylogenetic tree was constructed using the maximum Pasimony method with software MEGA version 3.1 (Kumar, Tamura and Nei, 2004). The stability relationship was evaluated by a boot strap analysis of 1000 repication.

3.5.4 Determination of genomic DNA by DNA-DNA hybridization

DNA-DNA hybridization was determined by photobiotin labeling DNA using colorimetric method according to Tanasupawat et al. (1992). Purified DNA solution (1 mg/ml) was mixed with photobiotin solution (1 mg/ml) at a ratio 1:1.5 in an Eppendorf tube and the mixture was irradiated with a sunlamp for 30 min on ice. Subsequently, 100 µl of 0.1 M Tris-HCl buffer pH 9.0 and 100 µl butanol were added to the mixture to remove excess photobiotins. The upper layer was romoved. One hundred µl of n-butanol was added and mixed well and the upper layer was removed again. The biotinylated DNA solution was boiled for 15 min and immediately cooled on ice. The solution was sonicated for 3 min and dissolved with hybridization solution (Appendix A 1.3)

Single-strand DNA was fixed on a microplate well (Nunc-Immuno^{TM} Plate: MaxiSorp^{TM} surface) by boiling 100 µl of 0.1mg/ml DNA solution for 10 min and immediately cooling in ice. Subsequently, DNA was fixed by incubating at 37°C for 2 h. After incubation, the DNA solution was discarded. The hybrization solution mixture containing biotinylated DNA was added. The microdilution plate was incubated at hybridization temperature of 40°C for 15-18 h.

After hybridization, the microplate wells were washed three times with 200 µl of 0.2×SSC buffer. Two hundred µl of solution I (Appendix A 1.14) was added to microdilution wells and incubated at room temperature for 10 min. Solution I was discarded. Solution II (Appendix A 1.15) (100 µl) was added and incubated at 37°C for 30 min. After incubation, 200 µl of PBS was used to wash wells for 3 times. One hundred µl of solution III (Appendix A1.16) was added to microdilution wells and incubated at 37°C for 10 min. The enzyme reaction was stopped by adding 100 µl 2M H_2SO_4 (Appendix A 1.2) (Verlander, 1992). The absorbance was measured at 450 nm using Microplate Reader (Microplate Manager^{R} 4.0 Bio-Rad Laboratories, Hercules, U.S.A.). Percentage of DNA-DNA relatedness was calculated as described in Appendix B.

3.5.5 Chemotaxonomy

3.5.5.1 Diaminopimelic acid

Diaminopimelic acid (DAP) in peptidoglycan of cell wall was determined using thin layer chromatography (Komagato and Suzuki, 1987). Three mg of dried cell was hydrolyzed with 1 ml of 6 N HCl at 100°C for 3 h. The hydrolysate was filtered and dried with N_2, then dissolved in 1 ml of water and spotted on cellulose TLC plate (no. 1.05716, 20 cm×20 cm Merck KGoA, Darmstadt, Germany).

TLC plates were developed in the mixture of methanol-water-6N HCl-pyridine (80:26:4:10, v/v). The spots were detected by 0.5% ninhydrin solution in butanol followed by heating at 100°C for 5 min.

3.5.5.2 Menaquinones

Menaquinone was carried out according to Komagato and Suzuki, (1987). Dried cells (100-300 mg) were extracted with chloroform:methanol (2:1) overnight. The suspension was filtered and dried then dissolved in a small amount of acetone and was separated using a silica gel TLC plate (no. 1.05744,20 cm × 20 cm Merck KGaA, Darmstadt, Germany). TLC was developed with methanol-water-6N HCl-pyridine (80:26:4:10, v/v) and band of menaquinone was detected by a UV lamp (254 nm). The band of menaquinone was scraped and dissolved in acetone. The purified menaquinone sample was analyzed by HPLC (Agilent Technologies Inc, Palo Alto, CA, USA) using a Hypersil BDS-C_{18} (100 × 4.0 mm) and detected at 270 nm.

3.5.5.3 Cellular fatty acids

Dried cells (40mg) were extracted in screw cap tube with 1.2 N NaOH in 50% methanol. The tube was mixed and heated at 100°C for 30 min and was cooled to room temperature. After heating, 2 ml of 6 N HCl in methanol was added to the suspension and was mixed for 5-10 sec. The samples were heated at 80°C for 10 min. and cooled to room temperature. After cooling, samples were added 1.25 ml of hexane-methyl tert-butyl ether (1:1) and were mixed well for 10 min. The upper layer was transferred to another tube and 3 ml of 0.3 N NaOH was added into the sample and mixed well for 5 min. The upper layer was analyzed by GC with a 5% phenyl-methyl silicone capillary column (0.2 mm × 25mm) with a flame ionization detector.

3.5.5.4 Genomic DNA G+C content

The DNA G+C content was followed the method of Tamaoka and Komagata (1984). The purified DNA was boiled at 100°C for 10 min and cooled immediately in ice for 5 min. Then, DNA was hydrolyzed to nucleotides using Nuclease P1 at 37°C for 1 h and hydrolyzed to nucleosides by alkaline phosphatase at 37°C for 1 h. The hydrolysate was analyzed by HPLC (Agilent technologies Inc, Palo Alto, CA, USA) using a Cosmosil $5C_{18}$ (150×4.6 mm) column and detected at 270 nm.

3.6 Characteristics of milk-clotting enzymes

3.6.1 Enzyme production and preparation

Virgibacillus sp. SK1-3-7 was cultivated in Y-medium containing 2.5% NaCl and incubated at 37°C for 4 days. After cultivation, crude enzyme was collected by centrifugation at 10,000×g for 30 min at 4°C. The crude enzyme was filtered through 0.45-µm membrane (Whatman, Kent, UK). The filtrate was added to attain concentration of 80%ammonium sulfate. The precipitate was centrifuged at 10,000×g for 30 min and decanted. The pellets were dissolved in 50 mM Tris-HCl , pH 7.5, and were dialyzed using dialysis membrane with molecular weight cut-off (MWCO) of 10 kDa (Pierce Chemical Company, Rockford, IL USA) at 4°C overnight against the same buffer. Purification was achieved using ÄKTA™ FPLC with UNICRON™ software version 3.2 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Sourec Q (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was packed in a column with 30 ml of bed volume. The enzyme was loaded into Source Q column (2.6×5.0 cm) equilibrated with 0.015 M sodium acetate buffer pH 5.0 (Buffer A) and was eluted with 0-1 M NaCl gradient in buffer. Five ml fractions were collected at a flow rate of

1 ml/min. Protein was detected at 280 nm. Fractions with clotting activity were pooled and diafiltrated against 50 mM Tris-HCl (pH 7.5) using a membrane with MWCO of 10 kDa (Vivaspin, Sartorius AG, Goettingen, Germany). The pooled fraction was referred to partially-purified enzyme and used for characterization.

3.6.2 Effect of pH on milk clotting activity

The activity of enzyme was measured at different pHs using 0.2 M buffer solutions: acetate (pH5.5-6.0), Tris-maleate (pH 6.5-7.0), Tris-HCl (pH 7.5-9.0) at 37 °C. Skim milk (10%) was dissolved in the respective buffer and adjusted to the set pH with 0.1N NaOH or 0.1 M HCl with rapid stirring. Each pH, a control was carried out without the enzyme. Milk-clotting activity was determined as described above.

For hydrolysis of skim milk at various pHs, 10% skim milk solution in 0.01 M $CaCl_2$ (0.1 ml) was used as a substrate and was hydrolyzed with 150 U/ml of partially-purifed enzyme at pH 5.5-7.0 for 2.5 min. The reaction was stopped by adding 5% SDS and heating at 100°C for 5 min. Protein content was determined by Lowry method (Lowry, Rosenbrough, Farr and Randall, 1951) using bovine serum albumin as a standard. Protein degradation was analyzed using 15% T sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were loaded into acrylamide gel at 10 μg per well. Electrophoresis was performed at 100 V. After electrophoresis, gel was stained with Coomassie Blue R-250 and destained by 25% ethanol and 10% acetic acid.

3.6.3 Effect of temperature on milk clotting activity

For optimum temperature, milk-clotting activity was determined at various temperatures of 25-70°C in 0.2 M acetate buffer (pH 5.5). Hydrolysis of skim milk at various temperatures were tested using 10% skim milk in 0.01 M $CaCl_2$ (0.1 ml) as a

substrate. Hydrolysis was carried out using 150 U/ml of partially-purified enzyme at different temperatures (30-75°C) for 2.5 min. The reaction was stopped by adding 5% SDS and heating at 100°C for 5 min. Degradation pattern was carried out as described above.

Oligopeptide content of skim milk hydrolysis was carried out as skim milk hydrolysis pattern except for the inactivate reaction which was stopped by adding 5% TCA and supernatant was collected by centrifugation at 10000 ×g for 10 min at 4°C. TCA-soluble oligopeptide contents were determined by Lowry method (Lowry, Rosenbrough, Farr, and Randall, 1951) using tyrosine (Sigma-Aldrich, St. Louis, MO., USA) as a standard. Blanks were prepared in the same manner except that inactivated proteinase by heating at 90°C for 5 min was used.

Thermal stability of partially-puried enzyme was investigated by incubating the enzyme at different temperatures (30-70°C) with 10°C interval for 10 and 30 min in 0.2 M Tris-maleate buffer pH 6.5. When incubation time was reached, samples were immediately cooled in ice. Subsequently, the remaining milk-clotting activity was determined and the activity of enzyme without incubation was taken to be 100%.

3.6.4 Effect of $CaCl_2$ concentration on milk-clotting activity

The effect of $CaCl_2$ concentration on milk-clotting activity of partially-puried enzyme was determined using skim milk containing various concentration of $CaCl_2$ (0, 5, 10, 20, 30, 50, 70 and 100 mM) in 0.2 M Tris-maleate buffer (pH 6.5) as a substrate. Milk-clotting activity was measured as described previously.

3.6.5 Hydrolysis of milk proteins

Hydrolysis of skim milk, whole milk and κ-casein (Sigma-Aldrich, St. Louis, MO., USA) was determined. Skim milk powder (Merck KGoA, Darmstadt, Germany)

was dissolved in 0.2 M potassium phosphate buffer pH 6.5 containing 0.01 M $CaCl_2$. Whole milk was obtained from Suranaree University of Technology (SUT) Farm. κ-Casein dissolved in 0.2 M potassium phosphate buffer, pH 6.5 at 10 mg/ml was used as a substrate. Commercial rennet from *Rhizomucor miehei* (Sigma Chemical Co., St. Louis, Mo, USA) or partially-purified enzyme (150 U/ml) was added to each protein solution and incubated at 37°C for different times (2.5, 5, 10, 20, 30 and 60 min).

Each time interval, the reaction was terminated by adding 5% SDS and heating at 100 °C for 5 min and protein content was determined by Bradford method (Bradford, 1961) using bovine serum albumin as a standard. Protein degradation was analyzed using SDS-PAGE. Skim milk and whole milk were loaded into gel at 10 μg per well, while κ-casien was loaded into gel at 15 μg per well. Electrophoresis was performed at 100V. After electrophoresis, gel was stained with Coomassie Blue R-250 and destained by 25% ethanol and 10% acetic acid.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of bacteria producing milk-clotting enzymes

Nineteen isolates of bacteria isolated from fish sauce fermentation were tested for screening the ability to produce milk-clotting enzyme. Screening for milk-clotting enzyme producing isolates was carried out using caseinate agar with skim milk as a substrate as shown in Figure 4.1 and Table 4.1. Only ten isolates (SK1-1-8, SK32, SK33, SK37, SK37-1, SK39, SK1-3-7, SK1-1-5, SKS20 and SK1-1-1) exhibited precipitation zone and clear zone on caseinate agar with a diameter ranging from 2.0-9.2 mm (Table 4.1). The precipitation zone was resulted from milk coagulation by the action of extracellular proteinases, while clear zone was formed by hydrolysis of casein. Thus, ten isolates were selected for further investigation on milk-clotting activities.

Proteolytic and milk-clotting activities of ten isolates were measured as shown in Table 4.2. Seven out of ten isolates (SK1-1-8, SK1-3-7, SK32, SK33, SK33, SK37, SK37-1 and SK39) were found to be milk-clotting enzyme producers based on the activity shown in Table 4.2. Crude enzymes from SK39 and SK1-3-7 showed the highest milk-clotting activity. In addition, SK39 showed the highest proteolytic activity. High proteolytic activity normally leads to lower yield, soft body and bitter taste of milk curd (Chazarra, Sidrach, López-Molina and Rodríguez-López, 2007; Vishwanatha, Appu Rao and Singh, 2010). Therefore, the ratio of milk-clotting

activity to proteolytic activity is used as an indicator for selecting the potential strain.

Many bacteria, including *Bacillus subtillis* (Dutt, Meghwanshi, Gupta and Saxena, 2008), *Enterococcus faecalis* TUA2495L (Sato, Tokuda, Koizumi and Nakanishi, 2004*), B. sphaericus* (El-Bendary, Moharam and Ali, 2007), *B. amyloliquefaciens* (He et al., 2011) are reported as a new source of milk-clotting enzyme for cheese making. These researchers have focused on isolating bacteria from environments that could express high milk-clotting activity and high ratio of milk-clotting activity to proteolytic activity. This is the first report of the milk-clotting enzyme producing isolates from fish sauce fermentation. According to Table 4.2, isolate SK1-3-7 showed the highest ratio of milk-clotting activity to proteinase activity. Therefore, it was selected for further investigation.

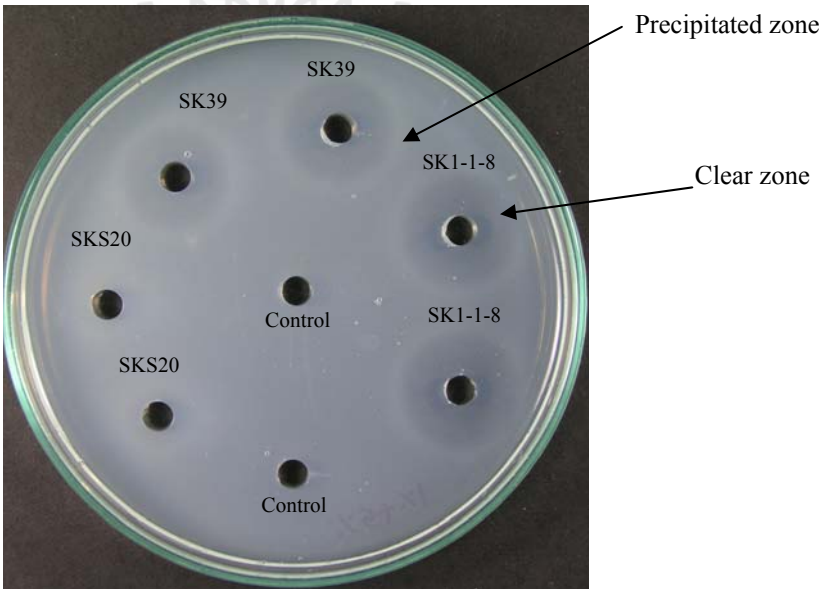

Figure 4.1 Milk-clotting activity and proteolytic activity on caseinate agar of bacteria isolated from fish sauce fermentation.

Table 4.1 Diameter of casein precipitation zone and clear zone after incubation with crude enzyme produced by various isolates at 37°C for 24 h.

Bacterial species	Bacterial isolate code	pH of crude extract[a]	Bacterial count (Log CFU/ml)	Zone diameter (mm.)[b]	
				Precipitation zone	Clear zone
Virgibacillus sp.	SK1-1-8	7.55	8.07	3±0.85	9±0.57
	SK32	7.21	7.95	2±0.71	8±0.71
	SK33	7.19	7.99	2±0.35	7.5±0.42
	SK37	7.58	7.79	3.5±1.41	7.5±1.84
	SK37-1	7.45	7.99	2±0.99	9±0.85
	SK39	6.89	8.0	2.5±0.85	7.5±0.71
	SK1-3-7	6.81	8.32	3±0.14	8±0.28
	SKW19	6.92	8.17	-	-
Brevibacillus sp.	SK35	7.4	7.70	-	-
Stapphylococcus sp.	SK1-1-5	6.27	7.96	3±0.41	7±1.41
	SK1-1-6	7.75	7.93	-	-
	SKS20	6.75	7.51	7±0.71	-
	SKW24-1	5.62	8.20	-	-
	SK 25	5.83	7.92	-	-
	SK 1-1-1	6.87	8.25	9.2±0.28	1.1±0.42
	SK 1-1-2	7.79	7.99	-	-
	SKW 23	7.13	8.23	-	-
	SKS 23	5.74	7.73	-	-
	SKW 29	5.21	8.27	-	-

-, No any zone

[a]Initial pH of Y-medium is 7.0

[b]Diameter of cork borer is 6.0 mm

Table 4.2 Proteolytic activity (PA) and milk-clotting activity (MCA) of crude extracellular proteinase by selected isolates.

Enzyme from isolate	Protein content (mg/ml)	Proteolytic activity (Unit/ml)	Specific activity (Unit/mg)	MCA Unit/ml	MCA/PA
SK37-1	1.32±0.11	0.77±0.03	0.58±0.02	21.57±0.08	28.13±1.35
SK1-3-7	1.57±0.01	0.98±0.02	0.62±0.01	46.84±0.08	47.89±1.05
SK32	1.52±0.14	0.24±0.01	0.16±0.01	7.34±0.00	30.32±1.52
SK33	1.71±0.08	0.69±0.04	0.40±0.04	25.67±0.02	37.48±2.05
SK37	1.27±0.03	0.65±0.01	0.51±0.00	25.66±0.01	39.53±0.88
SK1-1-8	1.55±0.01	0.72±0.03	0.47±0.02	31.87±0.11	44.08±1.88
SK39	1.38±0.02	0.98±0.02	0.71±0.02	43.86±0.26	44.62±0.45
SK1-1-1	3.14±0.08	0.02±0.00	0.01±0.00	-	-
SK1-1-5	3.11±0.04	0.09±0.01	0.03±0.00	-	-
SKS20	3.10±0.25	0.02±0.03	0.07±0.00	-	-

4.2 Identification of bacteria producing milk-clotting enzyme

4.2.1 Morphological and physiological characteristics

Three isolates, SK1-3-7, SK39 and SK1-1-8, having the highest ratio of milk-clotting activity to proteinase activity (Table 4.2) were selected for identification.

Three isolates were Gram-positive endospore-forming rods, occur singly, in pairs or short chains (Figure 4.2). Terminal and subterminal ellipsoidal spores were observed after cultivation on JCM 168 containing 5% NaCl at 35°C for 3-5 days (Figure 4.3). These isolates were facultative anaerobe. Colonies were circular, raised slightly transparent to opaque and white to cream color with 2-3 mm diameter after incubation for 2 days at 35°C on JCM 168 containing 5% NaCl.

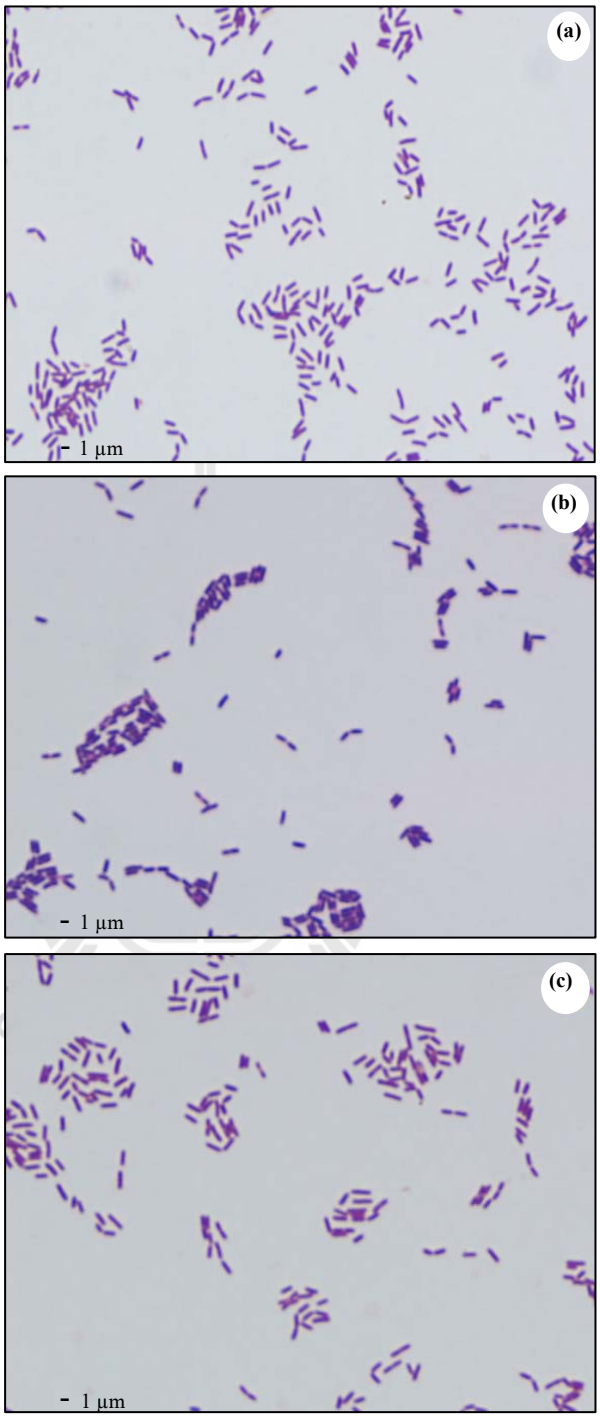

Figure 4.2 Gram staining of the selected isolates on JCM no.168 medium contained 5% NaCl and incubated at 35°C for 2 days, SK1-1-8 (a), SK39 (b) and SK1-3-7 (c).

(Bar=1 µm)

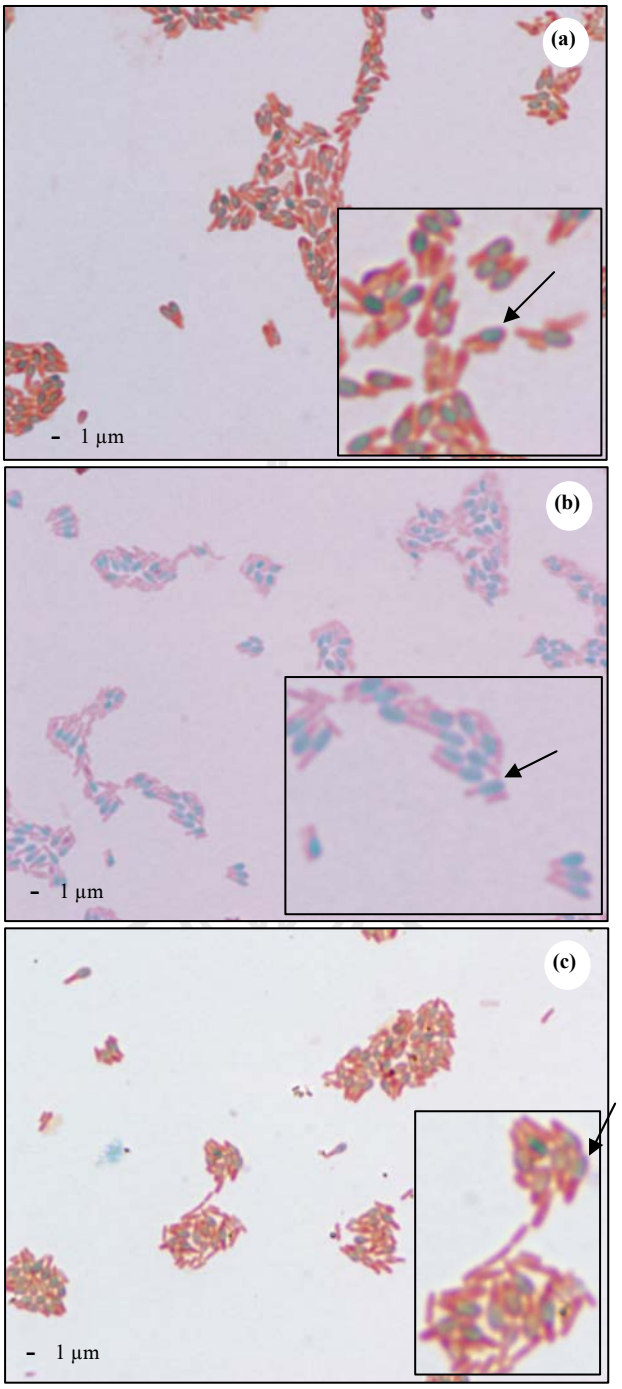

Figure 4.3 Endospores staining of the selected isolates on JCM no.168 medium contained 5% NaCl and incubated at 35°C for 3-5 days, SK1-1-8 (a), SK1-3-7 (b) and SK39 (c).

Bar=1 µm (The arrow demonstrated shape and position of spore)

Catalase and oxidase are positive (Table 4.3). They are moderate halophiles that grew at 0-20% NaCl with optimum NaCl concentrations of 5-15%. In contrast, *Virgibacillus halodenitrificans* is a moderate halophile that does not grow in the absence of salt but grow in the range of 0.5-20% NaCl (Table 4.3). SK1-3-7 and SK1-1-8 grew at temperature between 10-45°C with the optimum at 40°C, while SK39 had the optimal growth temperature at 35-40°C. Growth of SK39 and SK1-1-8 was observed at pH 6-10, while SK1-3-7 grew at pH 6-11. Optimum pH for growth of SK1-3-7 and SK1-1-8 was at pH 7, while optimum pH for growth of SK39 was at pH 6-7 (Table 4.3). They hydrolyzed casein and gelatin but not aesulin, starch and tweens 80. From the API 20E test kit, urease and hydrogen sulfide were not produced but nitrate was reduced by all isolates. Acid production from carbohydrates was shown in Table 4.3.

Table 4.3 Comparison of characteristics of the selected strains and *Virgibacillus holodenitrificans* ATCC 49067 (JCM 12304).

Characteristic	Bacterial isolate from fish sauce sample			Type culture strain	
	SK1-3-7	**SK39**	**SK1-1-8**	***Virgibacillus halodenitrificans* ATCC 49067[a]**	***Virgibacillus halodenitrificans* JCM 12304[b]**
Cell shape	Rods	Rods	Rods	Rods	Rods
Cell size (μm)	0.6-0.9 x 2.0-4.0	0.5-0.8 x 2.6-4.5	0.5-0.9 x 1.7-3.3	0.6–0.86x2.0–4.0	0.6–0.86x2.0–4.0
Spore shape	E	E	E	E	E
Spore position	S,T	S,T	S,T	S,T	S,T
Gram stain	+	+	+	V	V
Aerobic growth	+	+	+	+	+
Anaerobic growth	+	+	+	+	+
Growth at 0% NaCl (TSB pH 7.0)	+	+	+	-	-
0.5%	+	+	+	+	+
3%	+	+	+	+	+
5%	+	+	+	+	+
10%	+	+	+	+	+
15%	+	+	+	+	+
20%	+	+	+	+	+
25%	-	-	-	-	-
Optimum NaCl (%)	5-15	5-15	5-15	3-7	10

Table 4.3 (Continued)

Characteristic	Bacterial isolate from fish sauce sample			Type culture strain	
	SK1-3-7	**SK39**	**SK1-1-8**	***Virgibacillus halodenitrificans* ATCC 49067[a]**	***Virgibacillus halodenitrificans* JCM 12304[b]**
Growth at pH 5.0 (TSB 5% NaCl)	-	-	-	+	-
6.0	+	+	+	+	+
7.0	+	+	+	+	+
8.0	+	+	+	+	+
9.0	+	+	+	+	+
10.0	+	+	+	NA	+
11.0	+	-	-	NA	+
Optimum pH	7	6-7	7	7.5	7-8
Growth at temperature: (TSB 5% NaCl, pH 7.0)					
5°C	-	-	-	NA	-
10°C	+	+	+	+	+
15°C	+	+	+	+	+
20°C	+	+	+	+	+
25°C	+	+	+	+	+
30°C	+	+	+	+	+
40°C	+	+	+	+	+
45°C	+	+	+	+	+

Table 4.3 (Continued)

Characteristic	Bacterial isolate from fish sauce sample			Type culture strain	
	SK1-3-7	**SK39**	**SK1-1-8**	***Virgibacillus halodenitrificans* ATCC 49067[a]**	***Virgibacillus halodenitrificans* JCM 12304[b]**
50°C	-	-	-	-	-
Optimum temperature (°C)	40	35-40	40	35-40	35-45
Hydrolysis of:					
Aesculin	-	-	-	v (-)	-
Casein	+	+	+	+	+
Gelatin	+	+	+	+	+
Strach	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
H_2S production	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	-	-	-	+	+
Utilization of :					
D-Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
D-Fructose	+	+	+	v(+)	+
Sucrose	+	+	+	+	+

Table 4.3 (Continued)

Characteristic	Bacterial isolate from fish sauce sample			Type culture strain	
	SK1-3-7	**SK39**	**SK1-1-8**	***Virgibacillus halodenitrificans* ATCC 49067[a]**	***Virgibacillus halodenitrificans* JCM 12304[b]**
Acid production from:					
Glycerol	+	-	+	W	-
Erythritol	-	-	-	NA	-
D-Arabinose	-	-	-	NA	-
L-Arabinose	+	+	+	-	-
D-Ribose	+	+	+	+	-
D-Xylose	+	+	+	NA	-
L-Xylose	-	-	-	NA	-
D-Adonital	-	-	-	-	-
D-Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	-	-	-	NA	-
L-Rhamnose	+	+	+	-	-
Dulcitol	+	-	-	NA	-
Inositol	-	-	-	-	-
D-Mannitol	+	+	+	W	-
D-Sorbotol	+	+	+	NA	-

Table 4.3 (Continued)

Characteristic	Bacterial isolate from fish sauce sample			Type culture strain	
	SK1-3-7	SK39	SK1-1-8	*Virgibacillus halodenitrificans* ATCC 49067[a]	*Virgibacillus halodenitrificans* JCM 12304[b]
D-Maltose	-	-	-	+	+
D-Lactose	+	+	+	NA	-
D-Raffinose	+	+	+	-	-
D-Melibiose	+	+	+	-	-
D-Sucrose	+	+	+	NA	+
D-Trehalose	+	+	+	+	+
D-MeleZitose	-	-	-	-	-
Glycogen	-	-	-	-	-
Xylitol	-	-	-	NA	-
Gentiobiose	-	-	+	NA	-
D-Turanose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	NA	-
D-Fucose	-	-	+	+	-
L-Fucose	-	-	-	NA	-
D-Arabitol	-	-	-	NA	-
L-Arabitol	-	-	-	NA	-
Amygdalin	-	-	-	-	-
Arbutin	+	+	+	NA	-

Table 4.3 (Continued)

Characteristic	Bacterial isolate from fish sauce sample			Type culture strain	
	SK1-3-7	**SK39**	**SK1-1-8**	***Virgibacillus halodenitrificans* ATCC 49067[a]**	***Virgibacillus halodenitrificans* JCM 12304[b]**
Salicin	-	+	+	NA	-
Methyl-αD-mannoopyranoside	-	-	-	NA	-
Methyl-αD-Glucopyranoside	-	-	+	NA	-
N-acetylglucosamine	+	+	+	+	+
Potasssium gluconate	+	-	-	NA	-
Potasssium 2-Ketogluconate	-	-	-	NA	-
Potasssium 5-Ketogluconate	-	-	-	NA	-
Cell wall composition	meso-DAP	meso- DAP	meso-DAP	meso-DAP	meso-DAP
Major Manequinone	MK-7	MK-7	MK-7	MK-7	MK-7

[a]Phenotypic characteristics for type strain from Yoon et al. (2004)

[b]Phenotypic characteristics for type strain from the present study

E, Ellipsoidal; C, Central; S, Subterminal; T, Terminal; V, Variable; NA, Not available; W, Negative to weak

4.2.2 Chemotaxonomic characteristics

Isolate SK1-3-7, SK39 and SK1-1-8 contained *meso*-diaminomelic acid (*meso*-DAP) in cell wall peptidoglycan and MK-7 was the predominant menaquinone. This is similar to other members of the genus *Virgibacillus* (Heyndrickx et al., 1998; Heyrman et al., 2003). The G+C content of genomic DNA was 37.37, 37.06 and 36.46 mol%, respectively. *Virgibacillus halodenitificans* JCM 12304^T contains G+C content of 38.45 mol%. Cellular fatty acid profiles of these isolates showed dominant branched fatty acids (Table 4.4). The major cellular fatty acids of these isolates were anteiso-$C_{15:0}$ (59.4-65.4%) and anteiso-$C_{17:0}$ (24.7-29.5%) whereas those of *Virgibacillus halodenitificans* KCTC3790^T were anteiso-$C_{15:0}$ (51.8%), anteiso-$C_{17:0}$ (19.5%), iso-$C_{16:0}$ (11.8%) and iso-$C_{14:0}$ (7.4%) as shown in Table 4.5. Although cellular fatty acid components of 3 isolates were similar to *Virgibacillus* species, the proportion of individual fatty acid was different from *Virgibacillus halodenitrificans*^{T}.

Table 4.4 Cellular fatty acids of selected strains and *V.halodenitrificans* KCTC 3790^T.

	Content (% of Total)			
Fatty acid	**SK1-3-7**	**SK39**	**SK1-1-8**	***V. halodenitrificans* KCTC 3790[a]**
Saturated				
$C_{9:0}$	-	-	0.06	-
$C_{10:0}$	0.15	0.16	0.21	-
$C_{14:0}$	0.17	0.17	0.19	tr
$C_{15:0}$	-	0.33	0.47	tr
$C_{16:0}$	1.62	1.51	1.59	1.06
$C_{18:0}$	-	0.12	-	-

Table 4.4 (Continued)

Fatty acid	Content (% of Total)			
	SK1-3-7	SK39	SK1-1-8	*V. halodenitrificans* KCTC 3790[a]
Unsaturated				
$C_{16:1}\omega$ 7c alcohol	0.83	0.33	0.25	3.05
$C_{16:1}$ ω11c	0.59	0.16	0.15	0.4
Branched				
Iso-$C_{14:0}$	0.94	1.46	1.34	7.44
Iso-$C_{15:0}$	1.47	1.46	1.49	2.36
Iso-$C_{16:0}$	2.00	2.98	2.69	11.76
Iso-$C_{17:0}$	0.31	0.23	0.27	tr
Anteiso-$C_{13:0}$	-	0.11	0.11	-
Anteiso-$C_{15:0}$	59.41	65.42	64.54	51.8
Anteiso-$C_{17:0}$	29.45	24.7	26.06	19.52
Summed features*				
SF4	3.10	1.44	1.16	1.58

Note: tr=trace

*Summed features represent groups of two or three fatty acids that can not be separated. Summed Features4, Iso I $C_{17:1}$/Anteiso B $C_{17:1}$ and Anteiso B $C_{17:1}$/ Iso I $C_{17:1}$

[a] Component of cellular fatty acid of *V. halodenitrificans* KCTC 3790 from Lee et al. (2006)

4.2.3 Ribosomal rRNA gene sequence analysis

For 16S rRNA gene sequence analysis, these isolates were extracted and amplified using fD1/rP2 primer. The size of 16S rRNA gene fragments of the strains SK1-3-7, SK39 and SK1-1-8 was approximately 1526, 1533 and 1529 bp., respectively (Figure 4.4). SK1-3-7, SK1-1-8 and SK39 showed 99.4, 99.6 and 99.6% similarity with *V. halodenitrificans* DSM 10037^T (ATCC 49067^T, KCTC 3790^T and JCM 12304^T), respectively (Table 4.5). When phylogenetic tree analysis was

performed, the 3 selected strains fell in to the same cluster of *V. halodenitrificans* DSM 10037^T (Figure 4.5). Based on 16S rRNA gene sequence comparison all 3 isolates, were most closely related to *V. halodenitrificans*. However, these 3 strains had some characteristics different from the type strain. Phenotypic characteristics including the range of NaCl concentration and cellular fatty acid profiles were different from those of *V. halodenitrificans* (Table 4.3 and Table 4.4). All isolates grew at 0-20% NaCl and hydrolyzed casein, while *V. halodenitrificans* JCM 12304^T grew at 0.5-20% NaCl. In addition, all strains could be differentiated clearly from *V. halodenitrificans* JCM 12304^T based on fatty acid profiles. These strains contained significantly higher amount of anteiso-$C_{15:0}$ (59.41-65.42%) and anteiso-$C_{17:0}$ (24.7-29.45%) than *V. halodenitrificans*^{T}, but lower amount of Iso-$C_{16:0}$ (2.00-2.98%). Thus, genomic DNA-DNA relatedness was performed.

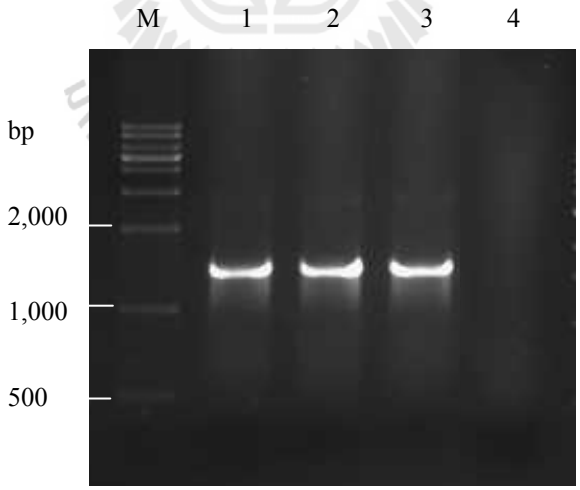

Figure 4.4 Gel electrophoresis of PCR products obtained from the amplification of bacterial 16S rDNA using primers fD1/rP2. Lane M, molecular weight markers; 1, SK1-3-7; 2, SK1-1-8; 3, SK39; and 4, negative control.

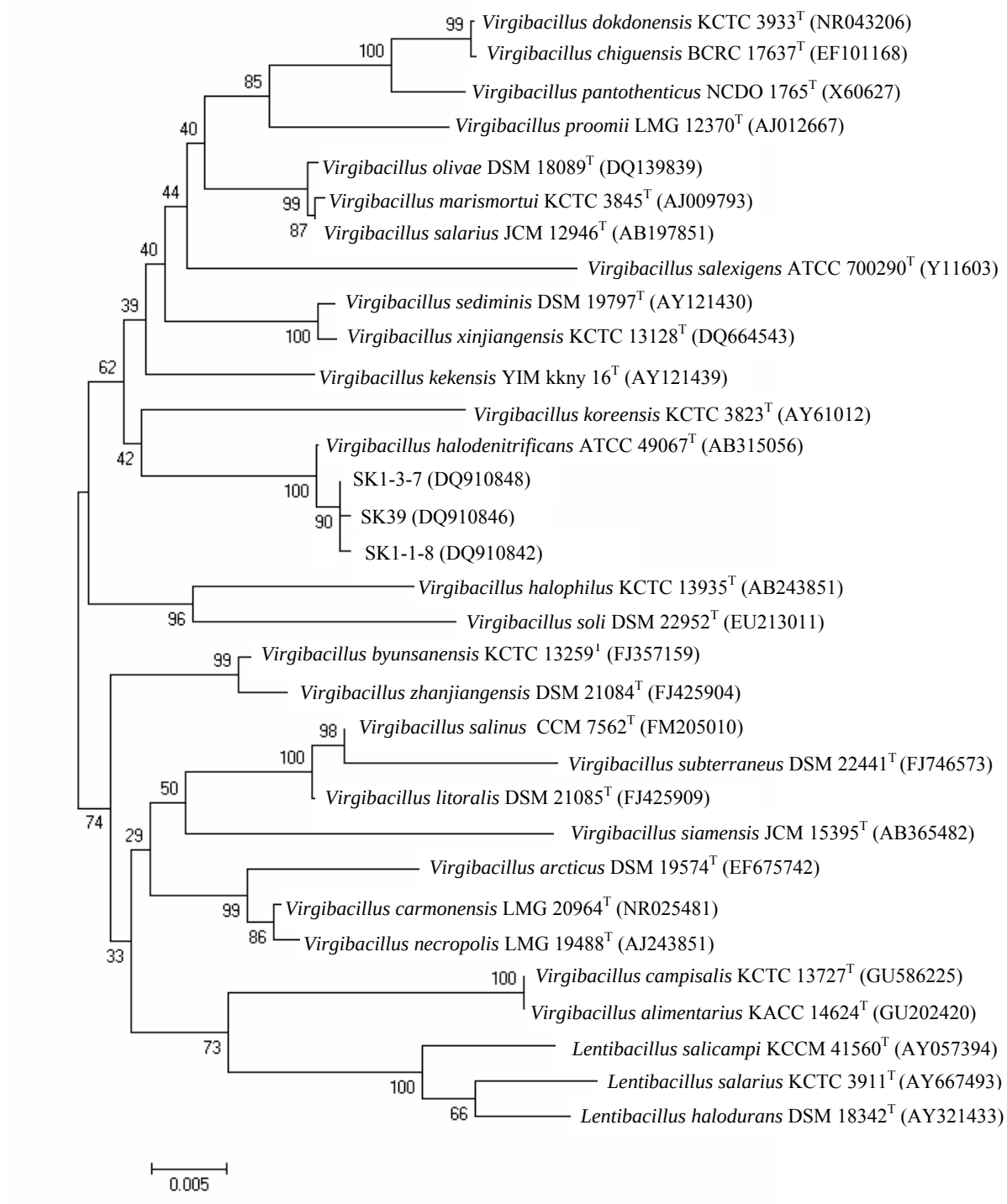

Figure 4.5 Phylogenetic tree of milk-clotting enzyme-producing bacteria isolated from fish sauce fermentation based on 16S rDNA sequence data.

Bar indicaties 0.005 substitutions per nucleotide position.

Table 4.5 Similarity of 16S rDNA sequence of *Virgibacillus* sp. SK1-3-7, SK1-1-8, SK39 and related species.

Bacterial	SK1-3-7	SK39	SK1-1-8	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	16	20	21	22	23	24	25	26	27	28	29
SK1-3-7	100																															
SK39	99.6	100																														
SK1-1-8	99.6	99.8	100																													
1	99.4	99.6	99.6	100																												
2	93.3	93.2	93.2	93	100																											
3	96.6	96.8	96.8	97	93	100																										
4	94.6	94.5	94.5	95	95	97	100																									
5	95.2	95.4	95.4	96	91	96	94	100																								
6	96.1	96.4	96.4	96	93	97	95	96	100																							
7	97	97.2	97.2	97	93	98	95	96	98	100																						
8	97.1	97.3	97.3	97	93	98	96	96	98	100	100																					
9	97	97.2	97.2	97	93	98	96	96	98	100	100	100																				
10	93.6	93.6	93.6	94	92	93	94	91	93	93	93	93	100																			
11	90.8	90.7	90.7	91	93	90	93	89	91	91	91	91	96	100																		
12	94.3	94.5	94.5	95	90	95	92	93	95	95	95	95	95	93	100																	
13	94.5	94.7	94.7	95	92	93	94	92	94	94	94	94	95	93	94	100																
14	95.9	96.1	96.1	96	91	95	93	93	96	96	96	95	94	91	95	99	100															
15	96.6	96.9	96.9	97	93	97	94	95	96	97	97	96	93	91	95	95	96	100														
16	89.7	89.7	89.7	90	90	89	91	88	89	89	89	89	90	88	88	90	89	92	100													
17	96.9	97.1	97.1	97	93	97	95	95	96	97	97	97	94	91	95	95	97	99	92	100												
18	96.7	96.9	96.9	97	93	96	94	95	96	97	97	97	94	91	95	95	96	98	91	98	100											
19	96.5	96.7	96.7	97	93	96	94	95	96	97	97	96	93	91	95	95	96	98	91	98	99	100										
20	96.4	96.6	96.6	97	92	96	94	95	96	97	97	97	94	91	96	95	96	97	91	97	97	97	100									
21	94.7	94.9	94.9	95	91	94	93	94	94	95	95	95	93	90	94	93	94	96	89	96	96	96	98	100								
22	94.4	94.3	94.3	95	94	94	96	93	93	94	94	94	95	93	93	95	93	95	93	95	95	95	97	96	100							
23	94.9	95.1	95.1	95	91	95	93	94	95	96	96	95	93	91	95	94	95	96	89	97	96	96	97	95	94	100						
24	95.9	96.1	96.1	96	92	96	94	94	96	96	96	96	94	91	95	94	95	96	89	96	96	96	96	94	94	95	100					
25	95.6	95.9	95.9	96	92	96	94	95	96	96	96	96	93	90	94	93	95	96	89	96	96	96	96	95	94	95	97	100				
26	96.9	97.1	97.1	97	92	97	95	95	97	98	98	98	94	91	95	94	95	97	90	97	97	97	97	95	94	96	96	96	100			
27	96.5	96.7	96.7	97	92	97	94	95	98	97	97	97	93	90	94	94	96	96	89	96	96	96	95	94	93	95	95	96	96	100		
28	94.6	94.6	94.6	95	94	95	96	93	96	95	95	95	95	92	92	95	94	94	90	94	94	94	94	92	95	93	93	94	94	98	100	
29	86.6	86.5	86.5	87	89	87	89	85	88	87	87	87	87	89	85	87	86	86	84	86	86	86	86	85	88	85	85	86	87	89	90	100

Note: 1, *Virgibacillus halodenitrificans* ATCC 49067^{T} (AB021186); 2, *Virgibacillus koreensis* KCTC 3823^{T} (AY616012); 3, *Virgibacillus sediminis* DSM 19797^{T} (AY121430); 4, *Virgibacillus xinjiangensisKCTC* 13128^{T} (DQ664543); 5, *Virgibaillus salexigens* ATCC 700290^{T} (Y11603); 6, *Virgibaillus proomii* LMG 12370^{T} (AJ012667); 7, *Virgibacillus marismortui* KCTC 3845^{T} (AJ009793); 8, *Virgibacillus salarius* (AB197851) JCM 12946^{T}; 9, *Virgibacillus olivae* DSM 18089^{T} (DQ139839); 10, *Lentibacillus halodurans* DSM 18342^{T} (AY321433); 11, *Lentibacillus salarius* KCTC 3911^{T} (AY667493); 12, *Lentibacillus halodurans* DSM 18342^{T} (AY321433); 13, *Virgibacillus alimentarius* KACC 14624^{T} (GU202420); 14, *Virgibacillus campisalis* KCTC 13727^{T} (GU586225); 15, *Virgibacillus necropolis* LMG19488^{T} (AJ243851); 16, *Virgibacillus arcticus* DSM 19574^{T} (EF675742); 17, *Virgibacillus carm*onensis LMG20964^{T}; 18, *Virgibacillus byunsanensis* KCTC 13259^{T} (FJ357159); 19, *Virgibacillus zhanjiangensis* DSM 21084^{T} (FJ425904); 20, *Virgibacillus litoralis* DSM 21085^{T} (FJ425909); 21, *Virgibacillus subterraneus* DSM 22441^{T} (FJ746573); 22, *Virgibacillus salinus* CCM 7562^{T} (FM205010); 23, *Virgibacillus siamensis* JCM 15395^{T} (AB365482); 24, *Virgibacillus halopilus* KCTC13935^{T} (AB243851); 25, *Virgibacillus soli* DSM 22952^{T} (EU213011); 26, *Virgibacillus kekensis* YIM kkny16^{T} (AY121439); 27, *Virgibacillus dokdonensis* KCTC 3933^{T} (NR_043206); 28, *Virgibacillus chiguensis* BCRC 17637^{T} (EF101168); 29, *Virgibacillus pantothenticus* NCDO1765^{T}

4.2.4 Genomic DNA-DNA relatedness

Genomic DNA of SK1-3-7, SK1-1-8 and SK39 was hybridized to genomic DNA of the reference strain, *V. halodenitrificans* JCM 12304^T. The result from DNA-DNA hybridization indicated that SK1-3-7, SK1-1-8 and SK39 were closely related among themselves with 91.22-107.02% relatedness, suggesting that these 3 isolates belonged to the same species (Table 4.6). However, they showed 38.96-45.85% DNA-DNA relatedness to *V. halodenitrificans* JCM 12304^T, which were below 70% cut-off (Wayne *et al*., 1987) for assigning to the same species. Therefore, these strains do not belong to *V. halodenitrificans*, and they are the novel species.

Table 4.6 DNA G+C content and genomic DNA-DNA relatedness of selected and type strains.

Strain	G+C content (mol %)	DNA-DNA relatedness (%) with			
		V.halodenitrificans JCM 12304^T	SK1-3-7	SK1-1-8	SK39
V.halodenitrificans JCM 12304^T	38.45	100	38.96	39.55	45.85
SK1-3-7	37.37	38.96	100.00	107.02	92.50
SK1-1-8	36.46	39.55	107.02	100.00	91.22
SK39	37.06	45.85	92.50	91.22	100.00

V. olivae sp. nov. showed the highest similarity of 16S rRNA gene sequence with *V. marismortui* (99%) (Quesada et al., 2007). However, the results from DNA/DNA hybridization of *V. olivae* showed low level of similarity between type strain of *V. marismortui* ATCC 700626^T (46.5%), which was well below the cut-off

value of 70% (Wayne et al., 1987). Wang et al. (2008) reported that *V. chiguensis* sp. nov. exhibited 16s rDNA sequence similarity of 99.6% to *V. dokdonensis*. However, the level of DNA-DNA relatedness between *V. dokdonensis* and *V. chiguensis* was only 17.5%. In addition, *V. chiguensis* grew at NaCl concentration up to 30%, which was higher than any *Virgibacillus* species previously described. Thus, Quesada et al. (2007) proposed *V. chiguensis* to be a novel species of the genus *Virgibacillus* based on DNA-DNA hybridization and phenotypic differences although the level of 16s rRNA gene sequence similarity was higher than the threshold value of 97%. Stackebrandt and Eber (2006) suggested to increase level of 16s rRNA gene sequence similarity from 97.0 to 98.7-99.0% for testing the genomic DNA-DNA relatedness for claiming a novel isolate, provided that clear phenotypic differences exist. On this basis, that SK1-3-7, SK39 and SK1-1-8 do not belong to any number of the 26 recognized *Virgibacillus* species.

Based on differences in phenotypic characteristics, chemotaxonomic characteristics and genomic DNA-DNA relatedness results, SK1-3-7, SK39 and SK1-1-8 do not belong to any recognized *Virgibacillus* species and are proposed to be a novel species of the genus *Virgibacillus*.

4.3 Characteristics of milk-clotting enzyme

4.3.1 Enzyme purification

Protein purification is a process that is used to isolate protein from complex mixture. This is vital for characterization of function, structure and interaction of the protein of interest. Partially-purified milk-clotting enzyme of *Virgibacillus* sp. SK1-3-7 was accomplished by two-step separation with 80% ammonium sulfate

($(NH_4)_2SO_4$) precipitation followed by Source 30Q anion exchanger chromatography. Results of the purification are summarized in Table 4.7. In ammonium sulfate precipitation, purity increased 1.1 fold with 44% recovery.

Table 4.7 Purification table of the milk-clotting enzyme of *Virgibacillus* sp. SK1-3-7.

Sample	Total Protein (mg)	Total milk-clotting activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude	2.85	23585	8275	1	100
80% $(NH_4)_2SO_4$ precipitation	1.10	10345	9421	1.1	44
Source 30Q	0.29	3960	13799	1.7	17

Two protein peaks were separated on Source 30Q anion exchange column, but only one peak with milk-clotting activity was resolved (Figure 4.6), with a 1.7-fold increase from crude enzyme and 17% recovery (Table 4.7).The purified milk-clotting enzyme showed 2 bands of 20 and 36 kDa (Figure 4.7). These values were different from those of fungal commercial rennet from *Rhizomucor miehei* and *R. pusillus*, which were reported to be 40.5 kDa and 49 kDa, respectively (Chitpinityol and Crabbe, 1998: Preetha and Boopathy, 1997). Moreover, it is noteworthy that most bacterial milk-clotting enzyme from *Bacillus amyloliquefaiens* D4, *B. subtilis* K-26 and *B. subtilis* YB-3 showed molecular mass of 58.2, 27 and 42 kDa, respectively (He et al., 2011; Rao and Mathur, 1979; Li et al., 2012). Proteinases from *Virgibacillus* sp. SK33 and SK37 showed proteolytic activity at molecular mass of 19, 32, 34 and 44 kDa (Sinsuwan, Rodtong and Yongsawatdigul, 2008, 2010: Phommao Rodtong and Yongsawatdigul, 2010), which were close to those of partially-purified milk-clotting

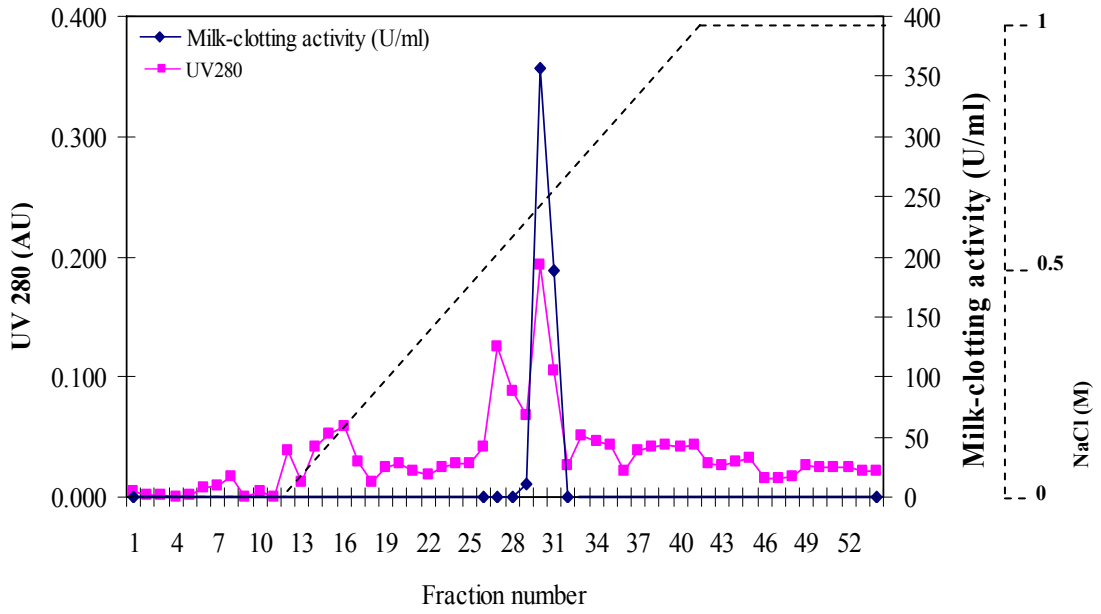

Figure 4.6 Chromatogram of the purified milk-clotting enzyme on Source 30Q column.

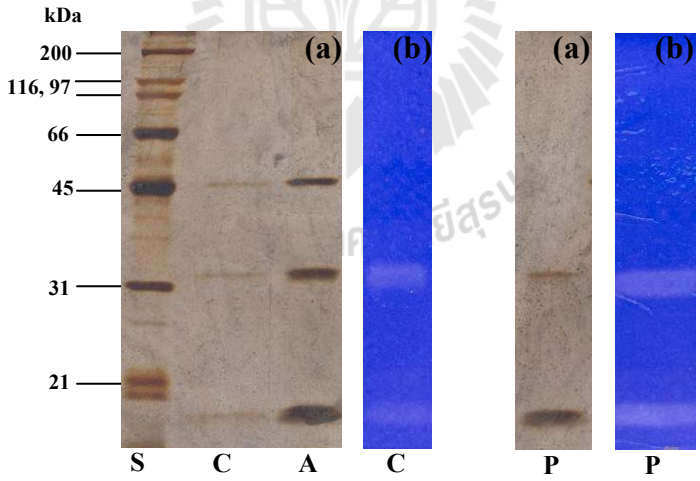

Figure 4.7 SDS-PAGE (12.5% T) of the partially-purified milk-clotting enzyme visualized by silver staining (a) and activity staining (b). S, standard molecular weight; C, crude enzyme; A, 80% $(NH_4)_2SO_4$ precipitation; P, Partially-purifed enzyme of *Virgibacillus* sp. SK1-3-7.

enzyme of *Virgibacillus* sp. SK1-3-7. *Virgibacillus* sp. SK33 showed 2 major proteinases with molecular mass of 19 and 32 kDa, while *Virgibacillus* sp. SK37 showed 3 major proteinases with molecular mass of 19, 34 and 44 kDa.

4.3.2 Effect of pH on milk clotting activity

Milk-clotting activity was not tested below pH 5.5 because skim milk protein underwent coagulation at low pH in the absence of enzyme. The control (in the absence of enzyme) at varied pHs did not coagulate. The milk-clotting activity decreased as pH increased from pH 5.5 to 9.5 with the pH optimum at 5.5. The enzyme was completely inactivated at pH 9.5 (Figure 4.8). Hydrolysis pattern of skim milk at pH 5.5-6.5 confirmed that coagulation at studied pHs was resulted from the action of proteinase. κ-Casein distributes throughout casein micelles and stabilizes casein micelles. When, κ-casein was preferably hydrolyzed, casein micelles became destabilization, leading to aggregation of casein micelles and formation of milk curd. The partially-purified enzyme showed the greater extent of κ-casein hydrolysis than the commercial rennet from *Rhizomucor miehei* (commercial rennet) (Figure 4.9). Moreover, the *Virgibacillus* sp. SK1-3-7 milk-clotting enzyme was active at a wide range of pH (5.5-6.5) compared to the commercial rennet. However, they also showed the same optimum activity at pH 5.5 (Figure 4.8 and 4.9). The partially-purified enzyme showed high milk-clotting activity at acidic pH. Hydrolysis pattern of skim milk was similar to the commercial rennet. Thus, it could be used as a rennet substitute for cheese making. Calf rennet exhibits the same pH-dependence being weaker in alkaline conditions than in acidic conditions with optimum at pH 3.6-3.7 (Richardson, Nelson, Lubnow, and Schwarberg, 1967). Milk-clotting enzymes from *B.*

amyloliquefaciens D4 (He et al, 2011), *Rhizopus oryzae* (Kumar, Sharma, Saharan and Singh, 2005), goat *(Capra hircus*) (Kumar, Sharma, Mohanty and Batish 2006) and glutinous rice wine mash liquor (Wang et al., 2009) showed similar optimum milk-clotting activity at pH 5.5.

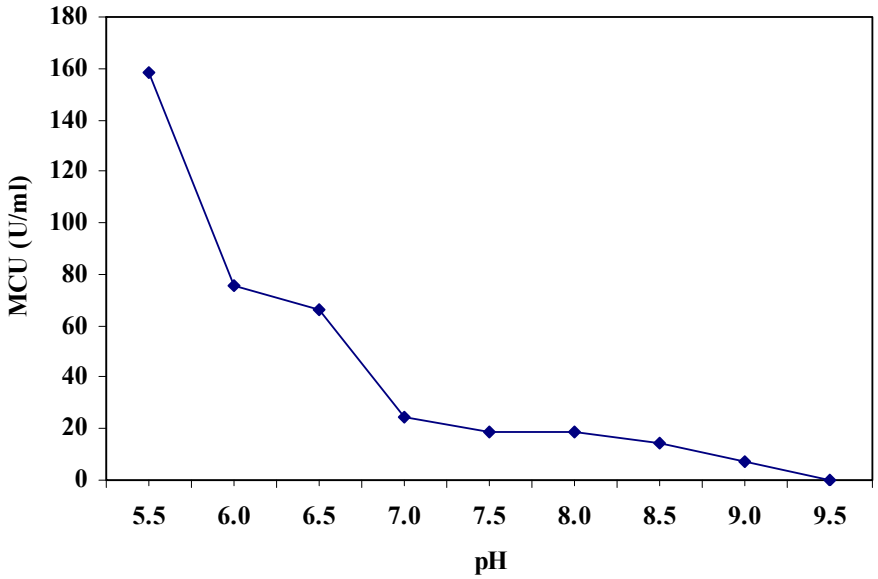

Figure 4.8 Effect of pH on the milk-clotting activity of partially-purified enzyme of *Virgibacillus* sp. SK1-3-7.

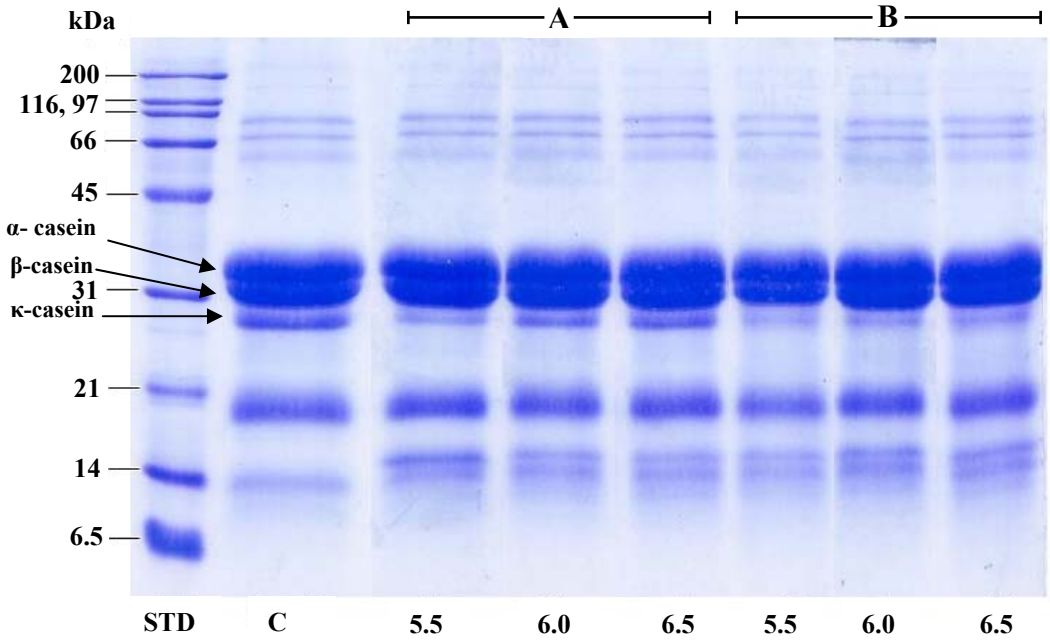

Figure 4.9 SDS-PAGE pattern of skim milk hydrolysis by *Rhizomucor miehei* (A) and partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 (B) at various pH values. STD, standard molecular weight; C, Control and numbers indicate pH values

4.3.3 Effect of temperature on milk clotting activity

The optimum temperature of milk-clotting ability was at 70°C. The milk-clotting activity increased as temperature increased from 25 to 70°C (Figure 4.10). This result indicates that enzyme is activated at relatively high temperature. The milk clotting enzyme from *Solanum dubium, Thermomucor indicae-seudaticae* N31 and *Bacillus subtilis* also showed optimum at 70°C (Mohamed Ahmed, Morishima, Babiker and Mori, 2009; Merheb-Dini, Gomes, Boscolo and da Silva 2010; Li et al., 2012). *B. megaterium* and *Penicillium oxalicum* showed high optimum temperature of milk-clotting activity at 65°C (El-Bendary, Moharam and Thanaa 2007; Hashem,

2000) and *Rhizopus oryzae* and *B. subtilis* K-26 showed optimum activity at 60°C (Kumar *et al*., 2005; Rao and Mathur, 1979)

Milk coagulation can be affected by both thermal treatment and enzymatic reaction. Skim milk hydrolysis and oligopeptide content at various temperatures were monitored in order to determine the cause of coagulation. κ-Casein was hydrolyzed at various temperatures as high as 75°C (Figure 4.11), producing a major peptide at 16 kDa. This result confirmed that coagulation at high temperature was resulted from the action of proteinase on κ-casein. Moreover, oligopeptide content of skim milk increased with increased temperature at the temperature range of 30-70°C (Figure 4.12). Oligopeptide content decreased at 75°C. The highest degree of hydrolysis was observed at 70°C. Based on these results, *Virgibacillus* sp SK1-3-7 proteinase optimally induced coagulation of skim milk at 70°C.

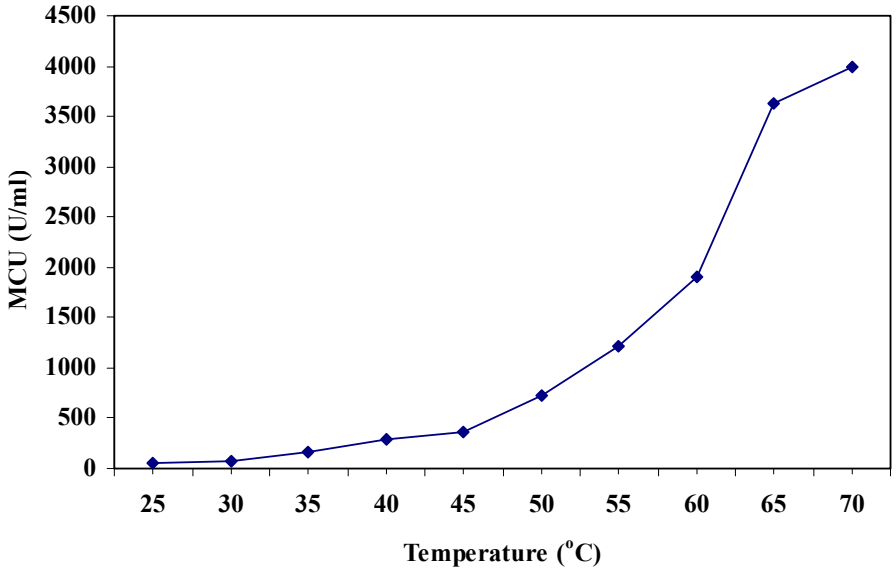

Figure 4.10 Effect of temperature on milk-clotting activity of partially purified enzyme of *Virgibacillus* sp. SK1-3-7.

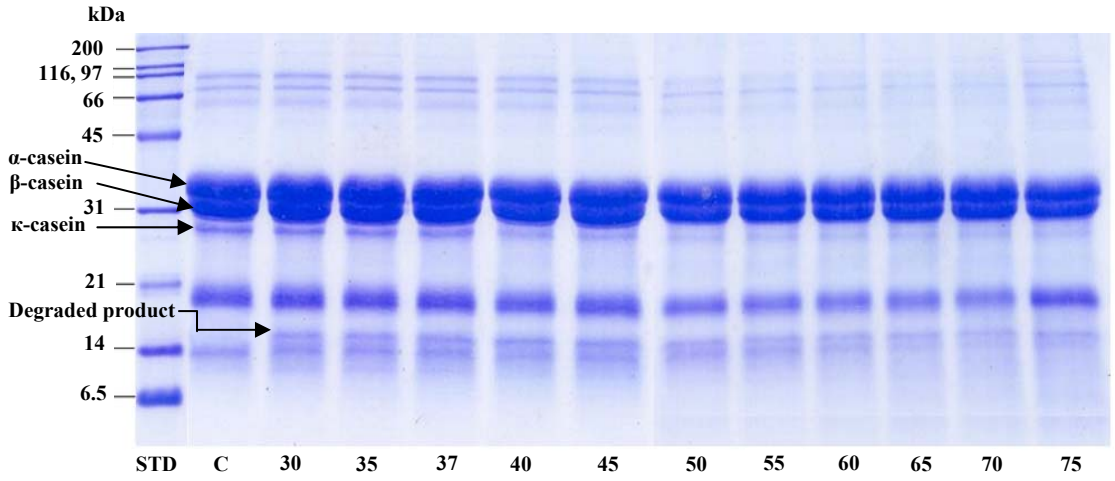

Figure 4.11 SDS-PAGE pattern of skim milk hydrolysis by partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 at various temperatures. STD, standard molecular weight; C, Control and numbers indicate temperature values

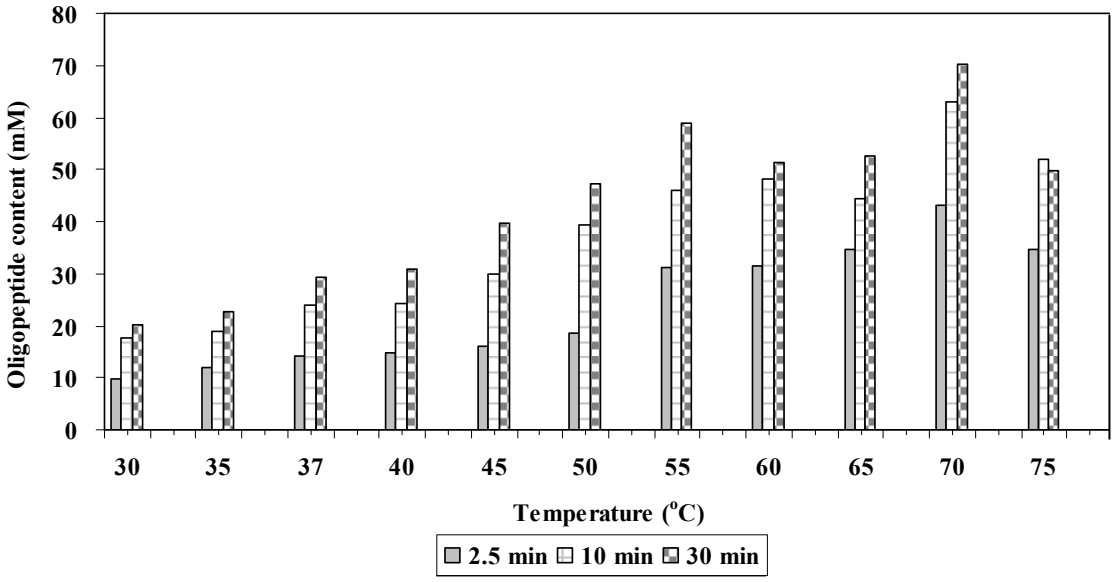

Figure 4.12 The extent of skim milk hydrolysis of partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 at various temperatures and time.

Effect of temperature on stability of the partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 is shown in Figure 4.13. The enzyme was stable up to 25-50°C and retained 100% activity. After incubation at 60°C for 10 and 30 min, activity reduced to70% and 90% of the original, respectively. Enzyme activity completely diminished at 70°C even 10 min incubation. The results indicated that partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 was sensitive to high temperature treatment. The commercial milk-clotting enzymes from animal and microbial sources

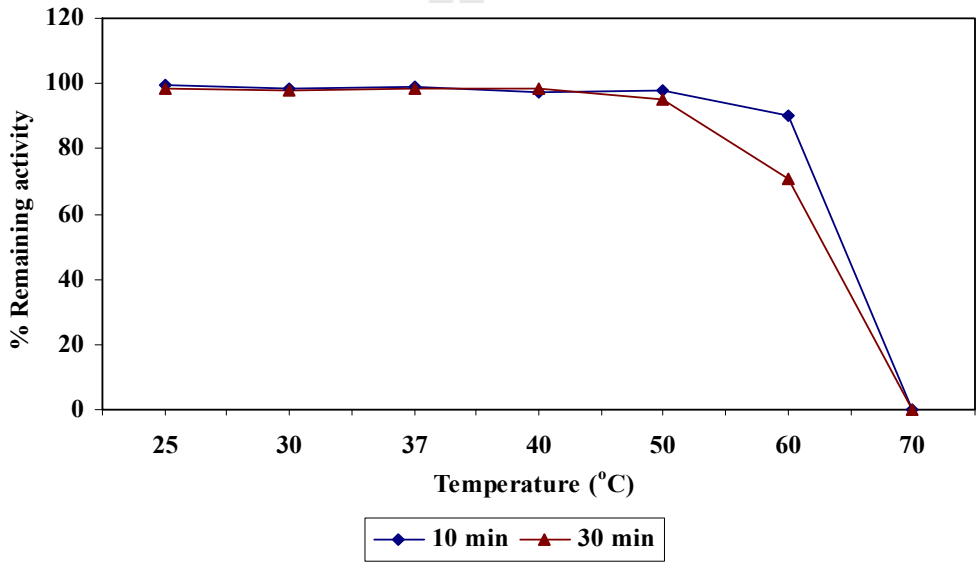

Figure 4.13 Effect of temperature on stability of partially-purified enzyme of *Virgibacillus* sp. SK1-3-7.

exhibit varied thermo-stability. Enzyme from *Rhizomucor miehei* is more thermoresistant with high stability at 60°C (Walsh and Li, 2005). Moschopoulou, Kandarakis, Alichanidis, and Anifantakis (2006) reported that calf chymosin became inactive at temperatures above 56°C. Milk-clotting enzyme from *B. subtilis* natto showed a decrease in activity after incubation at 70°C for 5 min (Shieh, Phan Tai and

Shih, 2009), which was similar to partially-purified enzyme of *Virgibacillus* sp. SK1-3-7. Remaining activity of milk-clotting enzyme added during cheese production is lost in whey and only 0–15% of activity remain in the curd (Sousa, Ardo, and Mcsweeney, 2001). The activity retained in the curd may result in excessive proteolysis, which lead to bitterness during the ripening stage (Sousa et al., 2001). Thus, the retention and activity of the milk-clotting enzyme in the curd are important and the use of enzymes that have higher thermal stability than the commercial rennet should be avoided to control the bitterness.

4.3.4 Effect of $CaCl_2$ on milk-clotting activity

Calcium has been known as an important substance for milk clot formation. Calcium induced coagulation of casein by acting as a bridge between casein micelles. An increase in calcium concentration leads to an increase in the coagulation rate (El-Bendary, Moharam, Ali, 2007; Kumar, Sharma, Saharan and Singh, 2005; Mohamed Ahmed, Babiker and Mori, 2010). Milk-clotting activity increased with $CaCl_2$ concentration up to 30 mM (Table 4.14). Increasing $CaCl_2$ concentration resulted in an enhancement of the clotting activity of the partially-purified enzyme from *Virgibacillus* sp. SK1-3-7. Ca^{2+} was found to be a potent activator of enzyme. Further increase of calcium led to a decrease in activity. The result was similar to milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341 (Vishwanatha, Appu Rao and Singh, 2010). Vairo-Cavalli, Claver, Priolo and Natalucci (2005) reported that milk-clotting activity of *Silybum marianum* flowers increased with calcium concentration with the maximum activity at 30 mM of $CaCl_2$. Sardinas (1968) reported that microbial rennin produced by *Endothia parasitica* showed optimum clotting activity

at 40 mM $CaCl_2$. Commercial rennet including *Rhizomucor miehei* and *R. pusillus* enzyme showed optimum activity at 20 mM $CaCl_2$ (Nouani et al., 2009). Ca^{2+} increased structural flexibility of the enzyme leading to an increase in enzyme activity. Some proteinases from halophiles required Ca^{2+} for activity. Proteinases from *Virgibacillus* sp. SK33 and SK 37 were also activated by calcium ions with an increased activity at $CaCl_2$ concentration ranging from 0-100 mM (Sinsuwan et al., 2008, 2010; Phommao et al., 2010). This result suggested that *Virgibacillus* enzyme required Ca^{2+} for milk-clotting activity. Raw milk contains about 10-11 mM $CaCl_2$ (Fox and McSweeney, 1998). Milk-clotting enzyme from *Virgibacillus* sp. SK1-3-7 is Ca^{2+}-activated enzyme. Thus *Virgibacillus* sp. SK1-3-7 enzyme might be useful for dairy industry as a milk-clotting agent.

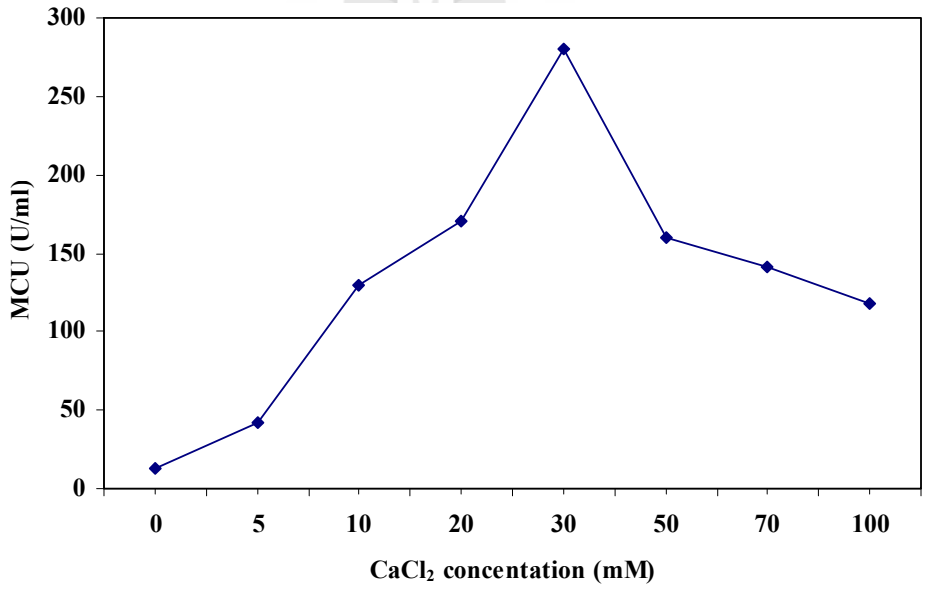

Figure 4.14 Effect of $CaCl_2$ on milk-clotting activity of partially-purified enzyme of *Virgibacillus* sp. SK1-3-7.

4.3.5 Hydrolysis of milk protein

Skim milk solution, whole milk and κ-caseins were hydrolyzed by partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 and compared to the hydrolysis pattern by the commercial rennet. SDS-PAGE of κ-casein hydrolysis is shown in Figure 4.15.

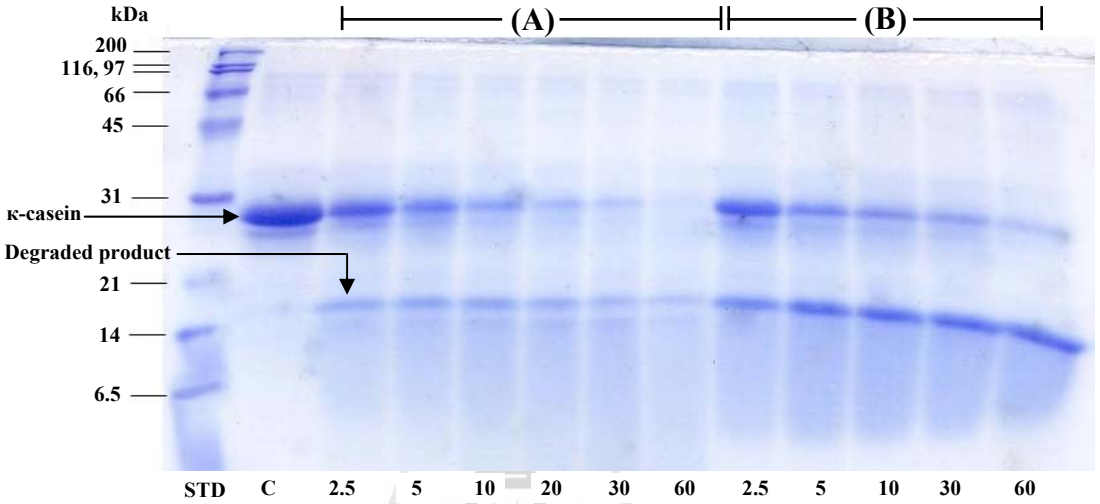

Figure 4.15 Hydrolytic patterns of κ-casein at 37°C for various incubation times. STD, standard molecular weight; Numbers indicate incubation time; Lanes labeled A and B indicate hydrolysis by partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 and *Rhizomucor miehei*, respectively.

Both partially-purified enzyme and the commercial rennet rapidly hydrolyzed κ-casein, producing a major band at 16 kDa after 2.5 min and assumed to be a para-κ-casein band (Egito et al., 2007; Mohamed Ahmed et al., 2010). Partially-purified enzyme produced bands with a molecular mass of 14.5 kDa after 5 min hydrolysis and completely hydrolyzed κ-casein after 60 min incubation. *Virgibacillus* sp. SK1-3-7 showed more proteolytic action on κ-casein than the commercial rennet. *Virgibacillus* sp. SK1-3-7 enzyme induced an extensive degradation of κ-casein and produced two

bands. Similarly, the Albizia seed protein extract hydrolyzed κ-casein to two bands with molecular mass of 16 and 17 kDa (Egito et al., 2007).

Degradation pattern of skim milk and pasteurized whole milk were observed on SDS-PAGE shown in Figure 4.16 a and b. A peptide with molecular mass of 16 kDa was evident in hydrolyzed samples, which was likely to be a para-κ-casein band. Para-κ-casein is a product generated from hydrolysis of κ-casein. *Virgibacillus* sp. SK1-3-7 enzyme showed similar hydrolytic pattern to the commercial rennet. α_{s}- and β-Casein were slightly hydrolyzed by both enzymes. α_{s}-, β- and κ-Casein are the main proteins found in cheese, which are known to affect yield, texture, and flavor of the cheese. Peptides and amino acids resulted from casein hydrolysis during cheese manufacturing and ripening processes are also important to yield and texture cheese (Li et al., 2012; Sousa et al., 2001). In skim milk solution, it was observed that κ-casein was completely hydrolyzed within 2.5 min (Figure 4.16 a), while complete hydrolysis of κ-casein in whole milk occurred after 5 min (Figure 4.16 b). This result suggested that skim milk is more susceptible to hydrolysis by partially-purified enzyme and commercial rennet than whole milk. It is concluded that partially-purified enzyme of SK1-3-7 was suitable for clotting skim milk and whole milk. Partially-purified enzyme showed low proteolytic action on casein, which was similar to commercial rennet. In addition, hydrolysis pattern of skim milk and pasteurized whole milk between these 2 enzymes were comparable. The partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 might be used as a rennet substitute.

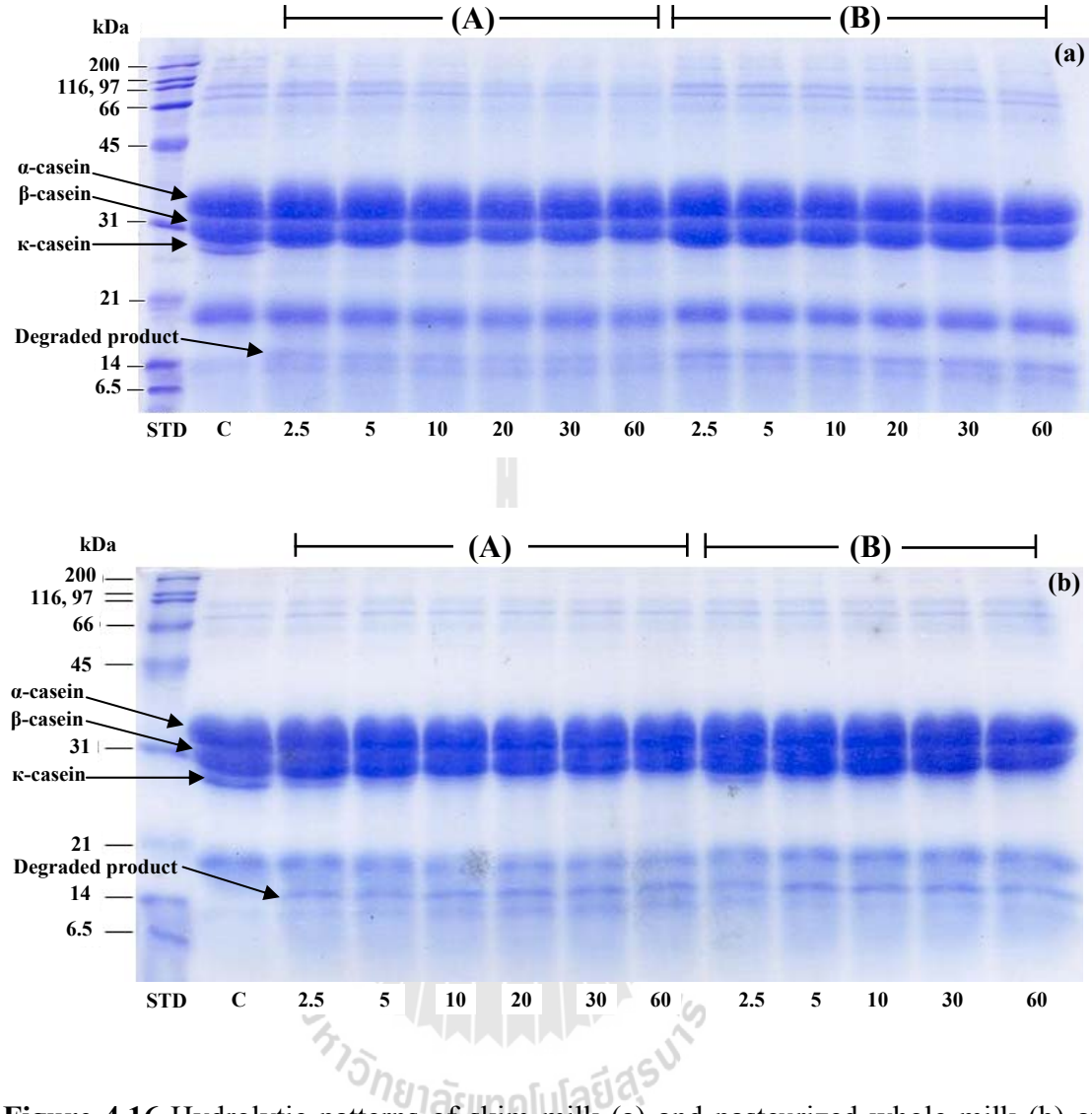

Figure 4.16 Hydrolytic patterns of skim milk (a) and pasteurized whole milk (b) at 37°C for various incubation times. STD, standard molecular weight; Number indicate incubation time; Lanes labeled A and B indicate hydrolysis by partially-purified enzyme of *Virgibacillus* sp. SK1-3-7 and *Rhizomucor miehei*, respectively.

CHAPTER V

CONCLUSIONS

Nineteen isolates of bacteria isolated from fish sauce fermentation were tested for screening the ability to produce milk-clotting enzyme. Ten isolates (SK1-1-8, SK32, SK33, SK37, SK37-1, SK39, SK1-3-7, SK1-1-5, SKS20 and SK1-1-1) exhibited precipitation zone and clear zone on casein agar. Based on milk-clotting activity, seven out of ten isolates (SK1-1-8, SK1-3-7, SK32, SK33, SK37, SK37-1 and SK39) were found to be a milk-clotting enzyme producer. Only 3 isolates (SK1-3-7, SK1-1-8 and SK 39) showed the highest ratio of milk-clotting activity to proteolytic activity and were selected for identification. All 3 isolates were Gram-positive rod and endospore-forming bacteria. They are moderate halophiles that grew at 0-20% NaCl with optimum NaCl concentrations of 5-15%. SK1-3-7 and SK1-1-8 grew at temperature between 10-45°C with the optimum at 40°C, while SK39 had the optimal growth temperature at 35-40°C. Growth of SK39 and SK1-1-8 was observed at pH 6-10, while SK1-3-7 grew at pH 6-11. Optimum pH for growth of SK1-3-7 and SK1-1-8 was at pH 7, while optimum pH for the growth of SK39 was at pH 6-7. They were identified as genus *Virgibacillus* based on phylogenetic analysis. Information from genomic DNA-DNA relatedness revealed that SK1-3-7, SK39 and SK1-1-8 belonged to the same species but do not belong to any recognized *Virgibacillus* species. They had several phenotypic characteristics that differentiated them from the type strain. Thus, they were proposed to be a novel species of the genus *Virgibacillus.*

Partially-purified milk-clotting enzyme of *Virgibacillus* sp. SK1-3-7 was accomplished by two-step purification with 80% ammonium sulfate ((NH4)2SO4) precipitation followed by Source 30Q anion exchanger chromatography and showed molecular mass of 20 and 36 kDa based on SDS-PAGE. The partially-purified of *Virgibacillus* sp. SK1-3-7 showed optimum temperature at 70°C and activity decreased when pH increased from pH 5.5 to 9.0. These enzymes were activated by CaCl2 showing maximal activity at 30 mM. Partially-purified enzyme was sensitive to high temperature treatment. Both *Virgibacillus* sp. SK1-3-7 enzyme and the commercial rennet rapidly hydrolyzed κ-casein, producing a major band at 16 kDa and assumed to be a para-κ-casein band. Moreover, partially-purified enzyme was suitable for clotting skim milk and pasteurized whole milk, and showed low proteolytic activity on casein, which was similar to the commercial rennet. Therefore, *Virgibacillus* sp. SK1-3-7 could be a potential source of milk-clotting enzyme with similar caseinolytic activity to that of the commercial enzyme.

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APPENDICES

APPENDIX A

Reagent and culture media preparation

1. Reagents

1.1 Crystal violet (Gram stain)

Crystal violet	2.00	g
Ethanol (95%)	20.00	ml
Mixed thoroughly		
Ammonium oxalate	80.00	ml
(1% Aqueous solution)		

1.2 H_2SO_4 (2M)

Conc. H_2SO_4	22.00	ml
Distilled water	178.00	ml

1.3 Hybridization solution (DNA-DNA hybridization)

Prehybridization solution	100.00	ml
Dextran sulfate	5.00	g

1.4 Hydrogen peroxide (3% solution)

Hydrogen peroxide	3.00	g
Distilled water	1,000.00	ml

1.5 Iodine solution (Gram stain)

Iodine	1.00	g

Potassium iodide	2.00	g
Add distilled water and bring volume up to	300.00	ml

1.6 Malachite green

Malachite green	5.00	ml
Distilled water	100.00	ml

1.7 Phosphate buffered saline (PBS 20x)

NaCl	160.00	g
KCl	4.00	g
Na_2HPO_4	23.00	g
KH_2PO_4	4.00	g
Add distilled water and brought volume up to	1,000.00	ml

1.8 Phenol:Chloroform (1:1 v/v)

Crystalline phenol was liquidified in water bath at 65˚C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a tight bottle.

1.9 Prehybridization solution (DNA-DNA hybridization)

100xDenhardt solution	5.00	ml
10 mg/ml Salmon sperm DNA	1.00	ml
20×SSC (20×Standard sodium citrate)	10.00	ml
Formamide	50.00	ml

Distilled water	34.00	ml

1.10 Safranin (Gram stain)

Safranin O (0.25% solution in 95% ethanol)	10.00	ml
Distilled water	90.00	ml

1.11 Salmon sperm DNA (10mg/ml)

Salmon sperm DNA	10.00	mg
TE buffer (10 mM, pH 7.6)	1.00	ml

Boiling for 10 min, immediately cooling in ice and sonication for 3 min.

1.12 Saline-EDTA (0.15 M NaCl + 0.1 M EDTA)

NaCl	8.76	g
EDTA (di-Sodium salt)	37.22	ml
Add distilled water and bring volume up to	1,000.00	ml

The medium was autoclaved at 121˚C for 15 min.

1.13 SDS (10% w/v)

Sodium dodecylsulfate (SDS)	100.00	g
Add distilled water and bring volume up to	1,000.00	ml

1.14 Solution I (DNA-DNA hybridization)

Bovine serum albumin (Fraction V)	0.25	g

Titron X-100	50.00	µl

1.15 Solution II (DNA-DNA hybridization)

Streptavidin-POD	1.00	µl
Solution I	4.00	ml

1.16 Solution III (DNA-DNA hybridization)

3,3',5,5'-Tetramethylbenzidine (TMB) (10 mg/ml in DMFO)	100.00	µl
0.3% H_2O_2	100.00	µl
0.4 M citric acid+0.2 M Na_2HPO_4 buffer pH 6.2 in 10% DMFO	100.00	µl

1.17 SSC (20×)

NaCl	175.30	g
Sodium citrate	88.20	g
Add distilled water and bring volume up to	1,000.0	ml

1.18 TE buffer (10mM Tris-HCl, 1mM EDTA)

Tris-HCl	0.79	g
EDTA (di-soduim salt)	0.37	g
Boric acid	5.54	g
Add distilled water and bring volume up to	1,000.0	ml

1.19 Tris-HCl buffer (0.1M, pH 9.0)

Tris	1.21	g
Distilled water	100.00	ml

Adjust to pH 9.0 using 1N HCl.

2. Culture media

2.1 O-F test medium (Atlas and Park, 1997)

Sodium chloride	5.0	g
Pancreatic digest of casein	2.0	g
di-Potassium hydrogen phosphate	0.30	g
Bromthymol blue	0.03	g
Agar	2.5	g
Glucose solution	100.0	ml
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2

The medium was autoclaved at 121˚C for 15 min.

2.2 Starch agar (Atlas and Park, 1997)

Soluble starch	2.0	g
Beef extract	3.0	g
Peptone	5.0	g
Sodium chloride	50.0	g
Agar	20.0	g
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2

The medium was autoclaved at 121˚C for 15 min.

2.3 Trypticase (tryptic) soy broth (TSB) (Atlas and Parks, 1997)

Tryptone	17.0	g
Phytone (Papaic digest soya meal)	3.0	g
Sodium chloride	5.0	g
di-Potassium hydrogen phosphate	2.5	g
Glucose	2.5	g
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2

The medium was autoclaved at 121˚C for 15 min.

2.4 Tween-80 agar (Atlas and Park, 1997)

Peptone	10.0	g
Sodium chloride	50.0	g
Calcium chloride	0.1	g
Yeast extract	2.5	g
Tween-80	10.0	ml
Agar	20.0	g
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2

The medium was autoclaved at 121˚C for 15 min.

APPENDIX B

Genomic DNA homology calculation

1. Genomic DNA homology calculation

$$\text{Homology (\%)} = \frac{\text{V sample} - \text{V reference}}{\text{V probe} - \text{V reference}} \times 100$$

V sample = Absorbance of sample at 450 nm

V reference = Absorbance of reference at 450 nm

V probe = Absorbance of probe at 450 nm

APPENDIX C

Nucleotide sequence alignment of 16S rRNA gene

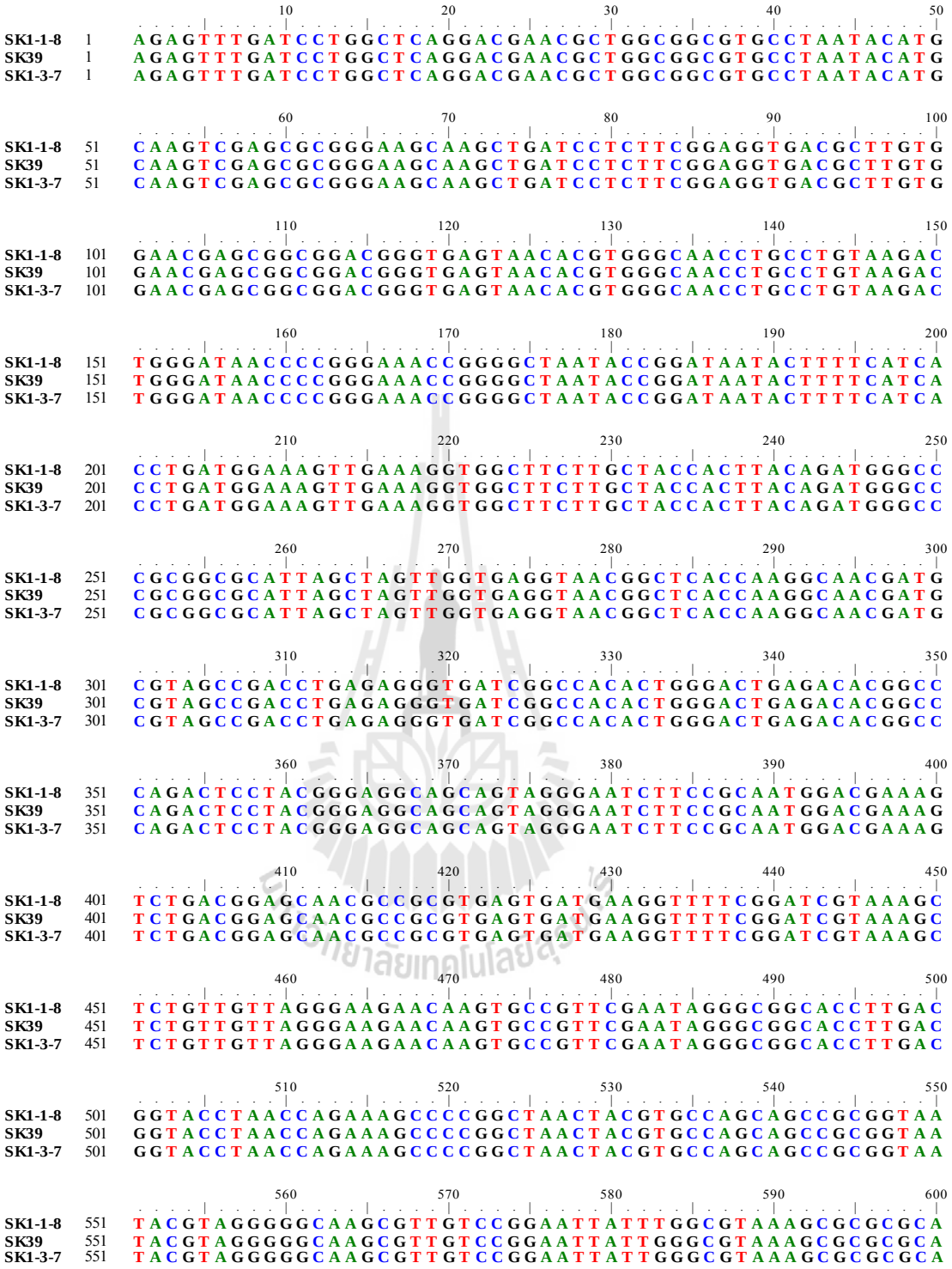

Figure 1C Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of milk-clotting enzyme-producing bacteria isolated from fish sauce fermentation.

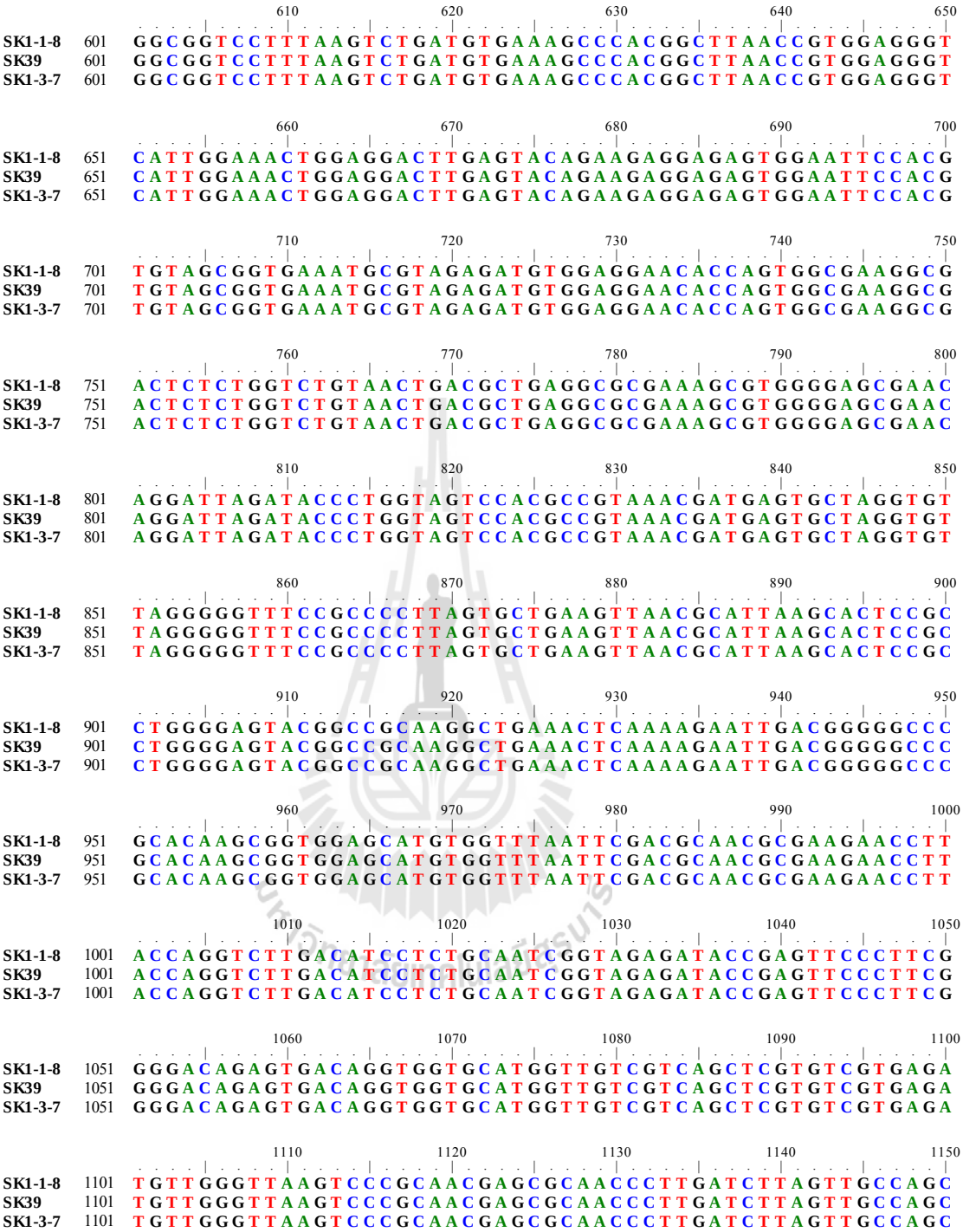

Figure 1C (continued) Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of milk-clotting enzyme-producing bacteria isolated from fish sauce fermentation.

Figure 4.5 (continued) Nucleotide sequence alignment of 16S rRNA gene (partial sequence) of milk-clotting enzyme-producing bacteria isolated from fish sauce fermentation.

APPENDIX D

Publication in GenBank (U.S.A.)

Table 1D Publication in GenBank (U.S.A.)

Isolation source	Bacterial isolate code	Nucleotide sequence submission		
		Identification result	Length of sequence	NCBI Accession no.
Sample from fish sauce fermentation process at 1st month	SK39	*Virgibacillus* sp. SK39	1533	DQ910842
	SK1-1-8	*Virgibacillus* sp. SK1-1-8	1529	DQ910846
Sample from fish sauce fermentation process at 3rd month	SK1-3-7	*Virgibacillus* sp. SK1-3-7	1526	DQ910848

APPENDIX E

Strains deposition at DSMZ (Germany)

Table 1 E Publication in DSMZ (Germany)

Isolation source	Bacterial isolate code	Nucleotide sequence submission		
		Identification result	**Length of sequence**	**DSMZ Accession no.**
Sample from fish sauce fermentation process at 1st month	SK39	*Virgibacillus* sp. SK39	1533	DSM 24893
	SK1-1-8	*Virgibacillus* sp. SK1-1-8	1529	DSM 24890

BIOGRAPHY

Sasitorn Kaewphuak was born in January 7th, 1985 at Bangkok, Thailand. She studied for her high school diploma (M.6) at Surathampitak School (2000-2002). In 2006, she received the degree of Bachelor of Science (Biotechnology) from Khon Kaen University, Khon Kaen, Thailand.

In 2007, she received scholarship from the National Science and Technology Development Agency (NSTDA), to study for a master degree in Food Technology at Suranaree University of Technology. Part of her thesis work was presented at a poster session at the 22nd Annual Meeting of the Thai Society for Biotechnology, October 20-22, 2010, Trang.