# Selection and Application of Protease- and Lipase-Producing Bacteria for Fermented Shrimp Paste (Kapi) Production

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**A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology (International Program)**
**Prince of Songkla University**
**2020**

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**Major Program** Biotechnology
**Academic Year** 2019

## ABSTRACT

Kapi is a Thai fermented shrimp paste which is widely consumed as a food condiment or flavoring in various Thai dishes. Generally, production of Kapi is mainly by a natural fermentation process which causes variations in the quality of the products. One effort that can be applied for improving the quality of Kapi is applying starter culture. This present study deals with the isolation, screening, and identification of protease- and lipase-producing bacteria from Kapi in conjunction with their technological properties and safety assessment to be applied as an autochthonous starter culture for Kapi production.

A total of 195 bacterial isolates exhibiting proteolytic activity on skim milk agar plate were obtained. Five bacterial strains (MSK-3P, MSK-4P, MSK-5P, MSK-7P, and MSK-10P) exhibited the high activity of protease (>5 U/mL) using Anson method were selected to be investigated for their potential application as starter culture. All the 5 strains were identified as *Virgibacillus halodenitrificans* by using 16S rRNA sequence analysis. All selected strains exhibited growth in the presence of NaCl up to 25%. Neither strains showed hemolytic activity nor biogenic amines (BAs) formation. Among all selected strains, only strain MSK-10P had no ability to form a biofilm, while other four strains showed a weak ability. Three strains, MSK-3P, MSK-4P, and MSK-10P showed susceptibility to all antibiotics tested however, strain MSK-7P exhibited multiple resistance to tetracyclin and vancomycin. According to the obtained results, *V. halodenitrificans* MSK-10P was selected to be applied as the starter culture for the production of Kapi.

A total of 46 bacterial isolates showing lipolytic activity on agar plate containing olive oil were selected. Among them, 4 selected bacterial strains, namely LSM3, LSM4, LSM15, and LSM16 exhibited the highest lipase activity using copper soap method were selected. Molecular identification by using 16S rRNA sequencing and was also confirmed by specific primer for staphylococci revealed that all of them were closely related to *Staphylococcus* sp. All of the selected isolates exhibited growth in the presence of NaCl up to 20%. In addition, strains LSM3, LSM15, and LSM16 showed non-hemolytic activity (γ-hemolysis), while all strains exhibited weak ability in biofilm formation. Strains LSM4, LSM15, and LSM16 displayed antagonistic activity toward only Gram-positive strains, *Staphylococcus aureus* DMST8840, *Bacillus cereus* DMST5540, and *Listeria monocytogenes* DMST17303. The antibacterial activity against tested Gram-positive bacteria came from bacteriocin-like inhibitory substance (BLIS). Moreover, isolates LSM15 and LSM16 showed susceptible to all tested antibiotics. Furthermore, classical staphylococcal enterotoxin (SE) genes were not found in all strains. Therefore, isolate LSM16 which exhibited the highest lipase activity was selected to be applied as the promising starter culture in the production of Kapi.

The optimization study for protease production by *V. halodenitrificans* MSK-10P was conducted by using -one-factor-at a-time- (OFAT) method. The optimum protease production was obtained at temperature 37$^{o}$C, pH 8.0, 1% (w/v) casein as substrate, 10% (w/v) NaCl, 2.5% (v/v) of inoculum, and under agitation at 150 rpm. In addition, time course production showed that the highest protease production was attained at 36 h of incubation which gave 3.83-fold increasing production, from 9.47 U/mL to 36.32 U/mL. Moreover, crude protease from *V. halodenitrificans* MSK-10P also showed halotolerance, since it can still active in 0-25% (w/v) NaCl.

The lipase production by *Staph. xylosus* LSM16 was also optimized by using OFAT method. The result showed that lipase production was optimum at temperature 37$^{o}$C, pH 7.0, 2% (w/v) olive oil, 10% (w/v) NaCl, 1.5% (v/v) of inoculum, under agitation at 150 rpm. Time course study for lipase production showed that the highest lipase production was achieved at 48 h, which gave 3.24-fold increasing of the

lipase production from 0.67 U/mL to 2.27 U/mL. As obtained in stability study towards salt condition, the crude lipase of *Staph. xylosus* LSM16 was found stable under 0-20% (w/v) NaCl.

The application of protease-producing bacteria, *V. halodenirificans* MSK-10P and lipase-producing bacteria, *Staph. xylosus* LSM16, was studied in the Kapi production. Four treatments of Kapi production were performed: (i) natural fermentation (as control, KC); (ii) inoculated with *V. halodenitrificans* MSK-10P (KP); (iii) inoculated with *S. xylosus* LSM16 (KL); and (iv) inoculated with both selected protease- and lipase-producing strain (KM). Physicochemical characteristic and microbiological safety of the Kapi were investigated. It was found that all the Kapi products inoculated with starter culture were meet the physicochemical characteristics and microbiological safety required by Thai Industrial Standard (TIS) 1205/2006. Moisture, salt, and total nitrogen content of Kapi products were in the range of 40.02-42.73%, 40.01-42.79%, and 6.33-7.82%, respectively. Microbiological quality of all finished Kapi product was also under limitations of criteria as regulated in TIS 1205/2006. In addition, none of the Kapi products contained the four important BAs (histamine, tyramine, putrescine, and cadaverine) mainly obtained in seafood samples. Moreover, sensory analysis also showed that all Kapi inoculated with starter culture were well-accepted by panelists. These results provided that *V. halodenitrificans* MSK-10P and *Staph. xylosus* LSM16 could be applied as promising strater cultures in the production of Kapi.

# ACKNOWLEDGEMENT

All of my praises, thanks, and worships to my Dear Lord Jesus, The Saviour of my soul, Amen.

I would like to express my heartfelt thanks to my advisor, Assoc. Prof. Dr. Suppasil Maneerat, for his high-quality supervisions, kindness, assistances, patiences, and valuable guidances throughout my Ph.D. study, conference, and publications. I also would like to extend a thousand thanks to Asst. Prof. Dr. Wasana Suyotha, as my co-advisor, for her warmth, kindness, admirable guidance, and supervisions throughout my study. I am very grateful to my examining committee: Asst. Prof. Dr. Apichat Upaichit, Prof. Dr. Duangporn Kantachote, and Prof. Dr. Wanna Choorit, for their kindness, useful comments, and valuable suggestions. I am also very grateful to all lecturers in Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, for their kind supporting and helping during my study.

I would like to express my sincere appreciation to Graduate School of Prince of Songkla University and Thai government, for providing the scholarship. Also, to the Ministry of Education and Culture of the Republic of Indonesia, and to the Rector of Sam Ratulangi University, Manado, for allowing me to pursue my study.

Finally, I am so grateful to my beloved husband (Yohanis), my children (Paul, Tabitha, and Catherine), my parents, and my big family for their warm love, prayers, supporting and understandings. Furthermore, my heartfelt thanks go to all of my lab mates in Department of Industrial Biotechnology, Faculty of Agro-Industry, Faculty of Agro-Industry, other PSU friends, Permitha friends, and my Christian family in Hat Yai, Songkhla, Thailand.

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# CHAPTER 1

# INTRODUCTION

## Introduction

Food fermentation undeniably implements a significant role in the food industry today. Almost one-third of total food consumption worldwide is fermented foods (Tamang and Kailasapathy, 2010). They are the hub where a group of indigenous microorganisms presented naturally in raw materials or starter cultures which convert the substrates into palatable foods accepted by the consumers (Holzapfel, 1997).

Fermented shrimp paste is one type of paste that is a popular food seasoning for the Southeast-Asian people. Kapi is a Thai fermented shrimp paste which is widely consumed as a food condiment or flavoring in various Thai dishes. Kapi is made from tiny shrimp or krill with sea salt in various concentrations between 10-30%. The mixture then was dried under the sunlight and fermented generally for several months according to local producer's recipes (Faithong and Benjakul, 2012). Other countries in Southeast-Asia are also familiar with this shrimp paste as additional popular ingredients in food. Various names of shrimp pastes in Southeast-Asia are *Bagoong* (Philippiness), *Belacan* (Malaysia), *Mắm tôm* (Vietnam), *Ngapi-yay* (Myanmar), and *Terasi* (Indonesia) (Hajeb and Jinap, 2012).

In general, production of Kapi is mainly by a natural fermentation process. This causes variations in the quality of the products. This spontaneous fermentation shows a large diversity of microorganisms. Variations of these microorganisms will cause a high probability of finding pathogenic microbes and toxic products. This certainly greatly affects the hygienic quality produced by Kapi so that it can endanger human's health.

Several factors that can affect the quality of the Kapi products are the type of shrimp used, the concentration of salt used, and the duration of fermentation (Prapasuwannakul and Suwannahong, 2015). Some researchers reported that those

variations noticeable the quality of Kapi. Wattanakul *et al.* (2011) found that most of 30 samples sold in Songkhla, Trang, and Pattalung provinces were not meet the quality requirement by Thai Industrial Standard (TIS) 1080-2535. Meanwhile, Pongsetkul *et al*. (2014) found that 11 samples from various locations and producers in Thailand showed various characteristics of proximate compositions, salt content, $A_w$, and pH. In addition, Kapi produced in less than 1 month would result in low-grade quality (Phitakphol and Kasetsat, 1995), while prolonged the fermentation time over 5 months cause not only the decreasing of amino nitrogen but also the increasing of ammonia level (Prapasuwannakul and Suwannahong, 2015).

One effort that can be applied for improving the quality of fermented food is applying starter cultures. By controlling food fermentation using starter cultures, it is expected that the domination of the original microflora could be achieved so that the fermentation can be expected to the desired attributes of the matrix and ensuring the nutritional quality and safety of foodstuffs as well. Therefore, the quality of fermented food can increase and meet the quality standards required by the regulation (Capozzi *et al.*, 2017; Marco *et al.*, 2017).

Preferably the starter culture used in food fermentation is the microbes which were selected and isolated from microflora contained in that fermented food because they would be able to adapt well to the conditions and environment in the fermentation process (Rebecchi *et al.*, 1998; Babic *et al*, 2011). In addition, those starter cultures were considered could dominate other microbiota in the fermentation process because they have special metabolic abilities (Leroy *et al.*, 2006). In fact, many microorganisms have been found in various fermented shrimp pastes by many researchers. Those microorganisms mostly identified as halo-tolerant and halophilic species belong to genera: *Bacillus*, *Lentibacillus*, *Oceanobacillus*, *Salinicoccus*, *Staphylococcus*, *Tetragenococcus*, and *Virgibacillus* (Kobayashi *et al.*, 2003; Pakdeeto *et al.*, 2007; Namwong *et al.*, 2009; Chuon *et al.*, 2014; Daroonpunt *et al.*, 2016). Nevertheless, none of the bacteria mentioned above have been characterized and applied as starter cultures in the fermented shrimp paste production.

The application of starter culture to improve the seafood fermentation process has been widely reported. Most of the starter cultures reported in seafood fermentation were protease-producing bacteria. *Virgibacillus* sp. SK 33, *Virgibacillus* sp. SK37 and *Staphylococcus* sp. SK1-1-5 possessed proteinase activity has been applied as the starter cultures in fish sauce production (Yongsawatdigul *et al.*, 2007) which contributed to reducing the fish sauce fermentation from 12 months to 4 months. Another report by Udomsil *et al.* (2011) who investigated that application of *Tetragenococcus halophilus*, isolated from fish sauce fermentation can also shorten the fermentation time to be 6 months. However, very few reports available for the application of protease-producing bacteria as starter culture in the production of fermented shrimp paste.

Lipase-producing bacteria were widely reported applied in the dairy and meat fermentation. The presence of coagulase negative staphylococci (CNS), such as *Staph. carnosus*, *Staph. equorum*, and *Staph. xylosus*, which possessed the lipolytic enzymes, has been reported to be positively contributed in the generation of specific flavor and aroma in the dairy and meat fermentation (Irlinger, 2008; Rantsiou *et al.*, 2005; Essid and Hassouna, 2013; Landeta *et al.*, 2013). Nevertheless, the possibility in application of lipase-producing bacteria in fishery and shrimp paste fermentation have been scarcely reported.

Before the application of the selected strain as a starter culture, the evaluation of its technological properties and safety-related attributes should be performed primarily. The final product should also be analyzed for its physicochemical characteristics, its safety, and its acceptance by the consumer. Therefore, this study dealed with the selection, evaluation, analysis, and application of the protease- and lipase-producing bacteria as promising starter culture in the Kapi production.

## Literature Reviews

### 1. Fermented shrimp paste

Fermented shrimp paste is a popular food condiment in many of Southeast-Asia dishes. Shrimp paste is known by different name according to the country, such as *Bagoong* (Philippiness), *Belacan* (Malaysia), *Kapi* (Thailand), *Mắm tôm* (Vietnam), *Ngapi-yay* (Myanmar), and *Terasi* (Indonesia) (Hajeb and Jinap, 2012). Mostly the shrimp paste was made from tiny shrimp or krill species, *Acetes* sp. or *Mesopodopsis* sp. Traditionally, in the Kapi production, the shrimp or krill mixed with a ratio salt to shrimp of 1:3-1:5, after which the mixture is sun-dried to obtain lower moisture content. The compacted paste then allowed to ripen until 3 months or more (Faithong *et al*., 2010). The final product of Kapi has pinkish to dark brownish color and varies from soft to hard solid paste (Phitakphol, 1993). Variations in type of shrimp raw material, the concentration of added salt, and the length of fermentation process causes the variety in characteristics and properties of the shrimp paste product (Peralta *et al*., 2008).

A regulation concerning the quality standard characteristics of Kapi product has been released by Thai Industrial Standard (TIS) is summarized in Table 1 below. Several researchers have been studied the characteristics and properties of Kapi product originated from various regions of Thailand (Table 2). However, it can be seen from the Table 2 that most of the Kapi product commercially available is below the TIS regulation.

**Table 1.** Parameter quality standard for Kapi (TIS 1205-2546)

| Parameter | Standard |
|---|---|
| *Microbiology standard* | |
| Coliform bacteria (MPN/g) | <3 |
| Fecal coliform (MPN/g) | - |
| *Eschericia coli* (MPN/g) | - |
| *Staphylococcus aureus* /0.1 g | None |
| *Clostridium perfringens* /0.01 g | None |
| *Salmonella Typhimurium* /25 g | None |
| *Vibrio parahaemolyticus* /0.1 g | - |
| Total plate count (CFU/g) | $<1 \times 10^5$ |
| Yeast and mold (CFU/g) | <50 |
| *Physicochemical standard* | |
| Moisture | ≤ 45% |
| pH | 6.5 – 7.8 |
| Salt content | ≥ 36% |
| Total nitrogen | ≥ 5.8% |

**Table 2.** Physicochemical characteristics of Kapi available commercially throughout local markets in Thailand.

| Samples | Moisture (%) | Protein (%) | Fat (%) | Carbohydrate (%) | Salt (%) | Aw | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| Kapi Trang | 43.33±6.97 | 22.62±11.31 | n.d.[a] | n.d. | 11.95±1.03 | 0.73±0.02 | 6.73±0.22 | Wattanakul *et al*., 2011 |
| Kapi Satun | 35.36 ± 0.81 | 24.07 ± 0.34 (37.23 ± 0.52)[b] | 1.76 ± 0.07 (2.72 ± 0.11) | 14.7 ± 0.53 (22.75 ± 0.61) | 19.76 ± 0.21 (30.57 ± 0.30) | 0.669 ± 0.00 | 7.94 ± 0.01 | Pongsetkul *et al*., 2014 |
| Kapi Ranong | 47.65 ± 0.18 | 21.86 ± 0.09 (41.76 ± 0.16) | 1.33 ± 0.15 (2.54 ± 0.28) | 4.7 ± 0.12 (8.97 ± 0.38) | 19.65 ± 0.03 (30.41 ± 0.05) | 0.732 ± 0.00 | 8.14 ± 0.13 | Pongsetkul *et al*., 2014 |
| Kapi Krabi | 47.75 ± 0.12 | 24.69 ± 01e (47.26 ± 0.18) | 1.28 ± 0.10 (2.46 ± 0.20) | 5.75 ± 0.19 (10.99 ± 0.32) | 17.23 ± 0.100 (26.65 ± 015) | 0.734 ± 0.00 | 8.03 ± 0.12 | Pongsetkul *et al*., 2014 |
| Kapi Songkhla | 40.73 ± 0.07 | 25.23 ± 0.07 (42.57 ± 0.12) | 1.45 ± 0.06 (2.45 ± 0.10) | 2.91 ± 0.18 (4.90 ± 0.28) | 22.93 ± 0.15 (35.47 ± 0.23) | 0.712 ± 0.00 | 7.97 ± 0.03 | Pongsetkul *et al*., 2014 |
| Kapi Rayong | 38.23 ± 0.48 | 29.77 ± 0.05 (48.19 ± 0.08) | 1.40 ± 0.10 (2.27 ± 0.16) | 7.07 ± 0.21 (11.45 ± 0.17) | 19.50 ± 0.17 (30.17 ± 0.26) | 0.695 ± 0.00 | 7.02 ± 0.19 | Pongsetkul *et al*., 2014 |
| Kapi Chachoengsao | 33.79 ± 0.17 | 28.56 ± 0.01 (43.14 ± 0.02) | 1.36 ± 0.02 (2.06 ± 0.03) | 13.91 ± 0.15 (20.99 ± 0.04) | 17.49 ± 0.26 (27.05 ± 0.41) | 0.705 ± 0.00 | 7.59 ± 0.03 | Pongsetkul *et al*., 2014 |

**Table 2**. (Cont.)

| Samples | Moisture (%) | Protein (%) | Fat (%) | Carbohydrate (%) | Salt (%) | Aw | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| Kapi Samut Songkram | 46.68±0.30 | 21.5±0.34 (40.34±0.52) | 1.06±0.08 (1.99±0.19) | 8.57±0.48 (16.07±0.86) | 22.38±0.85 (41.97±1.25) | 0.73±0.004 | 7.31±0.03 | Prapasuwannakul and Suwannahong, 2015 |
| Kapi Ta Dam | n.d. | 29.9±0.2 | 2.1±0.0 | 18.4 | 13.0±0.1 | 0.62–0.63 | 7.2–7.3 | Kleekayai *et al*., 2015 |
| Kapi Ta Deang | n.d. | 27.0±0.4 | 2.9±0.0 | 16.5 | 14.7±0.2 | 0.62–0.63 | 7.2–7.4 | Kleekayai *et al*., 2015 |

[a] n.d.: not determined

[b] Numbers in parentheses show the value on dry basis.

## 2. Halophile microrganisms

### 2.1 Definition of halophilic microorgnisms

Although salt is required in all life forms, halophiles (comes from the Greek, *hal*, which means salt, and *philos*, which means loving) were distinguished according to their requirements of salt concentration. At first, Kushner *et al.* (1978) classified halophiles as follows: extreme halophile (grow optimally at 2.5–5.2 M salt), moderate halophile (grow optimally at 0.5–2.5 M salt), and halotolerants, which are able to optimally grow at high salt concentrations without any exact requirements of salt to grow. Recently, DasSarma and DasSarma (2010) classified halophile microorganisms as follows: (i) slight halophiles were grown optimally at 0.2-0.85 M (1-5%) NaCl; (ii) moderate halophiles were grown optimally at 0.85-3.4 M (5-20%); and (iii) extreme halophiles were grown optimally at 3.4-5.1 M (20-30%) NaCl; while non-halophiles were grown optimally in less than 0.2 M NaCl and halotolerant microorganisms can grow either in the absence of salt or in high salt concentration. However, there are no sharp limits concerning the minimum and maximum concentration of salt needed by microorganisms to be able to grow optimally.

### 2.2 Adaptation of halophilic microorganisms

The growth and survival of bacteria during various salt condition required crucial factors such as the cellular adaptation and osmotic adjustment to stress. Halophilic microrganisms develop two different adaptive strategies to overcome the osmotic pressure caused by the high salinity condition in their habitat. Extreme halophilic bacteria adopt the salt-in mechanism by accumulating salt inside the cell. In contrast, the moderate halophilic and halotolerant bacteria can grow optimally at low salt conditions and the absence of salt, respectively. Moderate halophilic and halotolerant bacteria often encounter variation of salt concentrations because of the surrounding environment. Therefore, they implement numerous strategies to overcome such variations of saline conditions (Tsuzuki *et al.*, 2011). At the low salinity conditions, the water will directly invade the cell, which will cause the cell respond with fast evade of small solutes, thus releasing physical stress. While at the high salinity

conditions, fast discharged of water is counteract by an intensification of several companionable solutes, such as glycine betaine, ectoine, proline, and trehalose (Krämer, 2010).

Adaptation at the molecular level is brought through alterations in protein sequences to sustain them in the extreme conditions of the environment (Tekaia *et al*., 2002; Brocchieri, 2004; Nath *et al*., 2012). Analysis of the protein sequences responsible for the stability and functioning of the halophilic proteins can lead to our understanding of adaptation and help in protein engineering (Madigan and Marrs, 1997). Studies have indicated the importance of amino acid composition and dipeptide composition, and physicochemical properties of the amino acid can distinguish the halophilic proteins with reference to their non-halophilic counterparts (Ebrahimie *et al*., 2011; Purohit and Singh, 2011; Zhang and Yi, 2013).

The halophilic bacteria that follow the "salt-out" strategy accumulate high intracellular concentrations of the compatible solutes, which play a significant role in halo adaptation, such as protectants of macromolecules, cells, tissues, and even organs (Da Costa *et al*., 1998; Welsh, 2000). The term *compatible solutes* itself supports the cell architecture without any detrimental effect (Brown, 1976; Da Costa *et al*., 1998). Osmoregulation mechanisms have been reported in extreme halophiles, such as *Halobacterium salinarium* (Leuko *et al*., 2009) and *Haloferax volcanii* (Bidle *et al*., 2008), as well as in moderate halophiles, *Halobacillus dabanensis* (Feng *et al*., 2006), *Chromohalobacter salexigens* (Oren *et al*., 2005), and *Halomonas elongata* (Cánovas *et al*., 2000). However, little is known about the proteins involved in osmoregulation of halotolerant microorganisms. However, there are some studies on the molecular mechanisms adapted by these microorganisms, such as *Escherichia coli* (Lamark *et al*., 1994), *Bacillus subtilis* (Boch *et al*., 1996), *B. stearothermophilus* (Zhang *et al*., 2008), *Staph. xylosus* (Rosenstein *et al*., 1999), *Thalassospira* (López-López *et al*., 2002), and *Marispirillum* (Lai *et al*., 2009). Over the years, researchers have come to find halotolerant bacteria fascinating because of their robust nature and application prospects over a wide range of salinity (0%–25%) (Raval *et al*., 2018).

### 2.3 *Virgibacillus* sp.

*Virgibacillus* firstly described by Heyndrickx *et al*. (1998) and further developed by Heyrman *et al*. (2003). This endospore-spore forming, and aerobic genus belong to the Bacillaceae family within the Firmicutes phylum. Nowadays, *Virgibacillus* comprises 27 species which has legitimately scientific name and two other species, proposed as '*V. zhanjiangensis*' (Peng *et al*., 2009) and '*V. natachei*' (Amziane *et al*., 2013), but not published legitimately yet (Table 3). Other genera which are phylogenetically related include *Cerasibacillus, Lentibacillus, Oceanobacillus, Ornithinibacillus,* and *Paucisalibacillus*. Most of *Virgibacillus* species are found in high salt environment, only few species are found in fermented foods, as can be seen in the Table 3.

Several species of *Virgibacillus* has been reported involved in food fermentation process. Two isolates namely *Virgibacillus* sp. SK33 and *Virgibacillus* sp. SK37 which were isolated from nam-pla, a Thai fish sauce, have been reported as the potential starter cultures in fish sauce production. The length of fish sauce production inoculated with these starter cultures could be reduced from 12 months to be 4 months (Sinsuwan *et al.*, 2007; Yongsawatdigul *et al.*, 2007). In addition, Guan *et al*. (2011) reported that *Virgibacillus* sp. might be the principal participating group in Jeotgal (a salted and fermented traditional Korean seafood) fermentation. Meanwhile, *V. halotolerans* was isolated from cream-cheese preparation from dairy industry at southern Germany (Seiler and Wenning, 2013).

**Table 3.** Strains, accession numbers of the 16S rRNA gene sequence and origin source of *Virgibacillus* species

| Name of species | Accession number | Origin | Reference |
|---|---|---|---|
| *V. pantothenticus* | D16275 | Soil samples in Southern England | Heyndrickx *et al*., 1998 |
| *V. proomi* | AJ012667 | Soil samples in Southern England | Heyndrickx *et al*., 1998 |
| *V. carmonensis* | AJ316302 | Mural paintings in Spain | Heyrman *et al*., 2003 |
| *V. marismortui* | AJ009793 | Dead Sea | Heyrman *et al*., 2003 |
| *V. necropolis* | AJ315056 | Mural paintings in Spain | Heyrman *et al*., 2003 |
| *V. salexigens* | Y11603 | Solar salterns in Spain | Heyrman *et al*., 2003 |
| *V. halodenitrificans* | AY543169 | Solar saltern in France | Yoon *et al*., 2004 |
| *V. dokdonensis* | AY822043 | Dokdo Island in Korea | Yoon *et al*., 2004 |
| *V. koreensis* | AY616012 | Yellow Sea in Korea | Lee *et al*., 2006 |
| *V. halophilus* | AB243851 | Field soil in Japan (Kakegawa) | An *et al*., 2007 |
| *V. olivae* | DQ139839 | Wastewater of olive processing in Spain | Quesada *et al*., 2007 |
| *V. chiguensis* | EF101168 | Salt field in Taiwan (Chigu) | Wang *et al*., 2008 |
| *V. kekensis* | AY121439 | Keke Salt Lake in North-West China | Chen *et al*., 2008 |
| *V. salaries* | AB197851 | Salt crust from Tunisia | Hua *et al*., 2008 |
| *V. arcticus* | EF675742 | Canadian high Arctic | Niederberger *et al*., 2009 |

**Table 3.** (Cont.)

| Name of species | Accession number | Origin | Reference |
|---|---|---|---|
| *V. litoralis* | FJ425909 | Naozhou Island in South China Sea | Chen *et al*., 2009 |
| *V. salinus* | FM205010 | Salt Lake in Mongolia | Carrasco *et al*., 2009 |
| *V. sediminis* | AY121430 | Keke Salt Lake in North-West China | Chen *et al*., 2009 |
| *V. xinjiangnensis* | DQ664543 | Salt Lake in China (Xin-jiang province) | Jeon *et al*., 2009 |
| *V. zhanjiangensis* | FJ425904 | Naozhou Island in South China Sea | Peng *et al*., 2009 |
| *V. byunsanensis* | FJ357159 | Yellow Sea in Korea | Yoon *et al*., 2010 |
| *V. siamensis* | AB365482 | Fermented fish (pla-ra) in Thailand | Tanasupawat *et al*., 2010 |
| *V. subterraneus* | FJ746573 | Qaidam basin in China | Wang *et al*., 2010 |
| *V. alimentarius* | GU202420 | Gizzard shad from Korea | Kim *et al*., 2011 |
| *V. soli* | EU213011 | Yang-Ming Mountain in Taiwan | Kampfer *et al*., 2011 |
| *V. albus* | JQ680032 | Salt Lake in China (Lop Nur) | Zhang *et al*., 2012 |
| *V. campisalis* | GU586225 | West coast of Korea | Lee *et al*., 2012 |
| *V. halotolerans* | HE577174 | Babaria (Germany) | Seiler and Wenning, 2013 |
| *V. natechei* | JX435821 | Salt Lake in Algeria | Amziane *et al*., 2013 |

### 2.4 *Staphylococcus* sp.

*Staphylococcus* comes from the word *staphyle*, which means the group of grapes and *coccus* means round seeds. These bacteria are often found as normal flora in the skin and mucous membranes of humans, animals, as well as in food, beverage, and environment (Kloos *et al.*, 1992; Place *et al*., 2002; Gotz *et al*., 2006). Based on several molecular methods, the genus *Staphylococcus* can be classified into 6 main groups: *Staph. aureus* group, *Staph. epidermidis* group, *Staph. saprophyticus* group, *Staph. intermedius* group, *Staph. sciuri* group, and *Staph.* simulans group (Kloos *et al*., 1992; Gotz *et al*., 2006). Currently, genus *Staphylococcus* comprises around 47 identifiable valid species (DSMZ, 2015) which were fall into 6 groups aforementioned.

*Staphylococcus* is a Gram-positive, which cells arrangement might be single, pair, tetrad, or like a grape (irregular clusters), non-motile, non-endospore forming, catalase positive, easy to grow on various media, its metabolism is active, and producing pigments that vary from white to dark yellow. Most of *Staphylococcus* species are facultative anaerobe, where some shows better growth in aerobic condition than anaerobic condition. In addition, most of *Staphylococcus* grow well around neutral pH, some are mesophilic (20-40$^{o}$C), and showed halotolerance (grow well at 10 % NaCl) (Kloos *et al.*, 2001).

Several strains of *Staphylococcus* have been widely found in fermented foods. Some important members of *Staphylococcus* involved in food fermentations are as follows:

- *Staph. nepalensis*, isolated from mackerel fish sauce, able to improve fish sauce odor (Fukami *et al.*, 2004). This strain grows well at 10% NaCl with optimum temperature at 30$^{o}$C (Fukami *et al*., 2004; Spergser *et al*., 2003).
- *Staph. carnosus*, isolated from fermented sausages and cured ham played significant role on the meat's flavor formation (Kloos and Wolfshohl, 1991; Hammes and Knauf, 1994). *Staph. carnosus* also involved in fermented fish and soy sauces in Asia (Tanasupawat *et al.*, 1991). It grows well at 6-15 % NaCl, pH range of 6.8-9.0, and temperature range is 18-42$^{o}$C (Kloos *et al.*, 2001).

- *Staph. piscifermentans*, isolated from Thai fermented fish product. It grows well at pH 6.8-9.0, 6-15% NaCl, and optimum temperature 42-45$^{o}$C (Tanasupawat *et al.*, 1992).
- *Staph. saprophyticus*, can be found in raw beef and pork, dairy product, cheese, dry fermented sausages, also in food utensils used in food processing (Rall *et al*., 2010; Coton *et al*., 2010; Soares *et al*., 2011). It grows well at pH 5.0-6.8, 20-40$^{o}$C, and 0-15% NaCl (Schleifer and Kloos, 1975).
- *Staph. xylosus*, widely isolated from fermented meat and its derivatives, low-salt fermented fish, and fermented fish sauce (Yongsawatdigul *et al*., 2007; Jeong *et al*., 2016; Chen *et al*., 2017). It grows well at pH 4.9-7.0, 20-40$^{o}$C, and 0-15% NaCl (Schleifer and Kloos, 1975).

## 3. Protease

Protease is a class of hydrolase enzyme which cleavage proteins into simpler molecules, such as oligopeptides or amino acids, by hydrolyzing the peptide bonds. This enzyme is important for all living things since it is essential in the metabolism of proteins, hormons, and neurotransmitters (Adler-Nissen, 1993; Poliana *et al*., 2007).

International Union of Biochemistry and Molecular Biology (IUBMB) classified proteases into subgroup 4 of group 3 (hydrolases). The EC divides proteases into exopeptidase groups (3.4.11-19) and endopeptidase (3.4.21-24). Exopeptidase works by hydrolyzing the release of a single amino acid at the N or C end, whereas endopeptidase acts on a specific polypeptide chain in a peptide bond. The endopeptidase group consists of serine protease (EC 3.4.21), cysteine protease (EC 3.4.22), aspartate protease (3.4.23), and metalloprotease (EC 3.4.24). On the active side of these enzymes are serine, cysteine, aspartic acid, and metal atom, respectively. Exopeptidases group divided into three subgroups, aminopeptidase, carboxypeptidase, and omega peptidase groups. Aminopeptidases attack the N-terminal group in the polypeptide chain to release amino acid residues. On the other hand, carboxypeptidases attack the C-terminal group of the polypeptide chain to release amino acids. Some mechanisms of protease hydrolysis are shown in Figure 1.

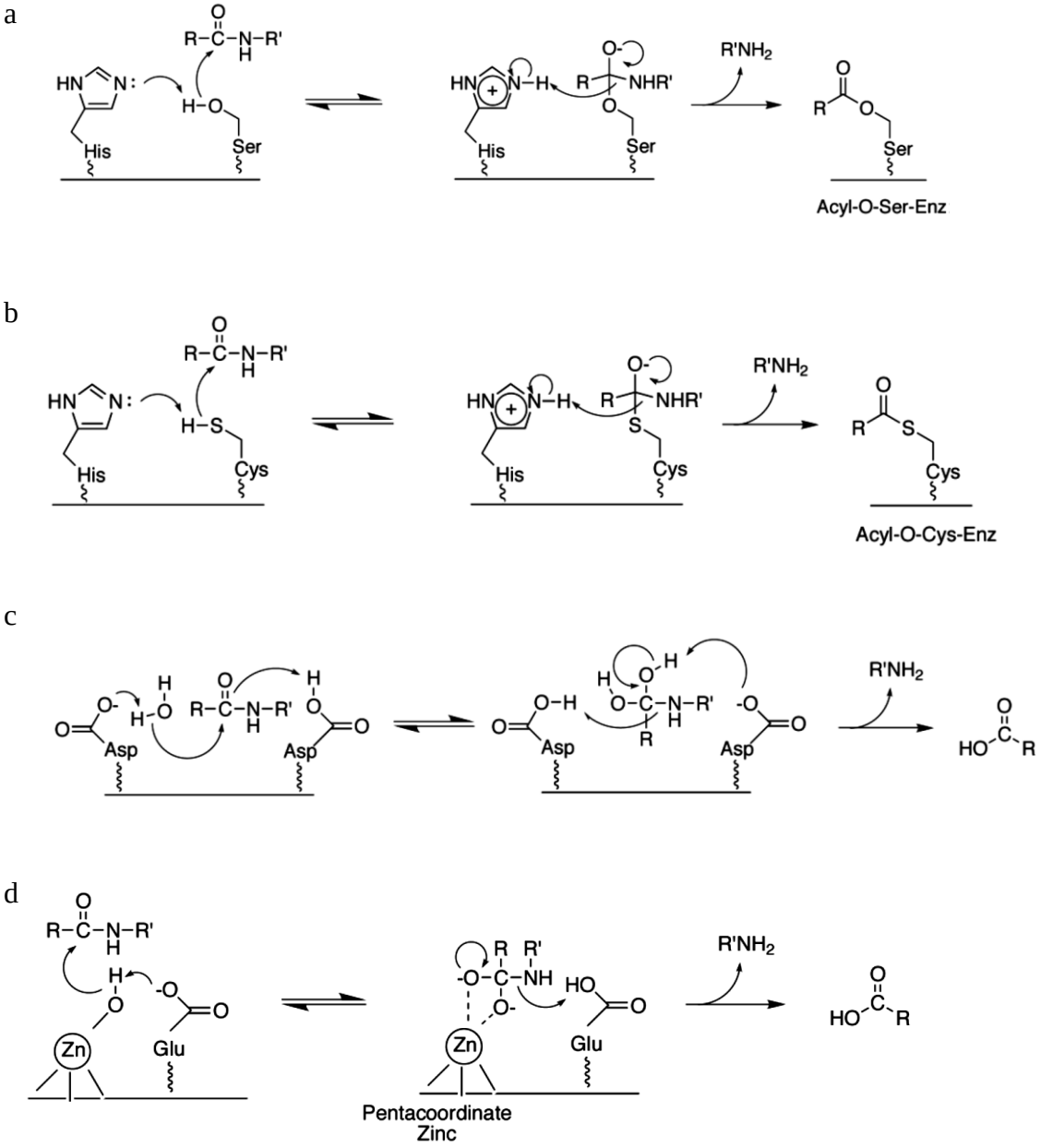



**Figure 1.** Four mechanistic variations of protease to activate a peptide bond for hydrolysis, by: a. Serin protease; b. cystein protease; c. aspartic protease; d. metalloprotease (Evans and Cravatt, 2006).

Microbial proteases naturally extracellular and directly produced in the medium of fermentation. Prominently genus *Bacillus* are the most dominant source of alkaline protease producers. Numerous *Bacillus* species from many different exotic

environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis*, *B. amyloliquifaciens*, and *B. mojavensis* (Rao *et al.*, 1998; Gupta *et al.*, 2002). Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity (Rao *et al.*, 1998).

## 4. Lipase

Lipases (E.C. 3.1.1.3) are enzymes which catalyze the hydrolysis reaction of insoluble acylglycerols, different with esterases whose substrates are soluble acylglycerols (Jaeger *et al.*, 1999). Lipases from different origins have similarity in its structure. Briefly, lipases are β–hydrolase with parallel β–strands as a core which is surrounded by α-helices (Figure 2). The catalytic triad was located in their core mixed β–sheet. The catalytic triad was composed of serine, histidine and aspartate or glutamate residues. Almost all lipases have a coverlid which comprises of amphiphilic peptide loop with the function to conceal the active site when in condition of inactive state. At the water-lipid interface, the lid undertakes a conformational change which may cause the substrates access the active site. This can be observed when lipase activity sharply increased if the substrate concentration is in emulsion form (Ollis *et al*., 1992; Jaeger *et al*., 1999).

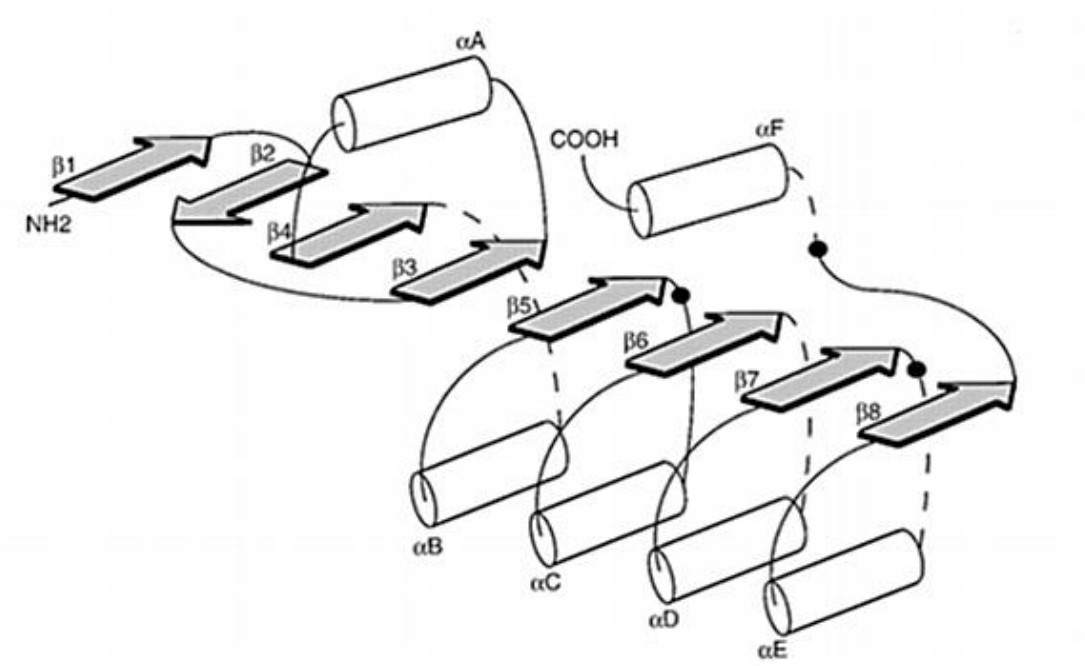



**Figure 2.** The canonical structure of the α/β hydrolase fold in lipase. α-Helices are represented by cylinders and β-sheets by arrows (Ollis *et al.*, 1992, Jaeger *et al.*, 1999).

Mechanism of lipase catalysis is depicted in Figure 3. The catalysis is carried out in three steps. Initially, the lipid substrate was bind and the serine residue was activated by neighboring histidine, then the carbonyl group of substrates was attack by the nucleophile oxygen atom of serine (Figure 3a). This mechanism leads to the second step, the transient acyl enzyme formation which was stabilized by an oxyanion hole and alcohol releasing from the substrate (Figure 3b). In the final step, an incoming water molecule attacked the carbonyl atom which attached to the serine, then the release of acyl product was induced, and the catalytic site was regenerated as well (Figure 3c) (Reis *et al.*, 2009).

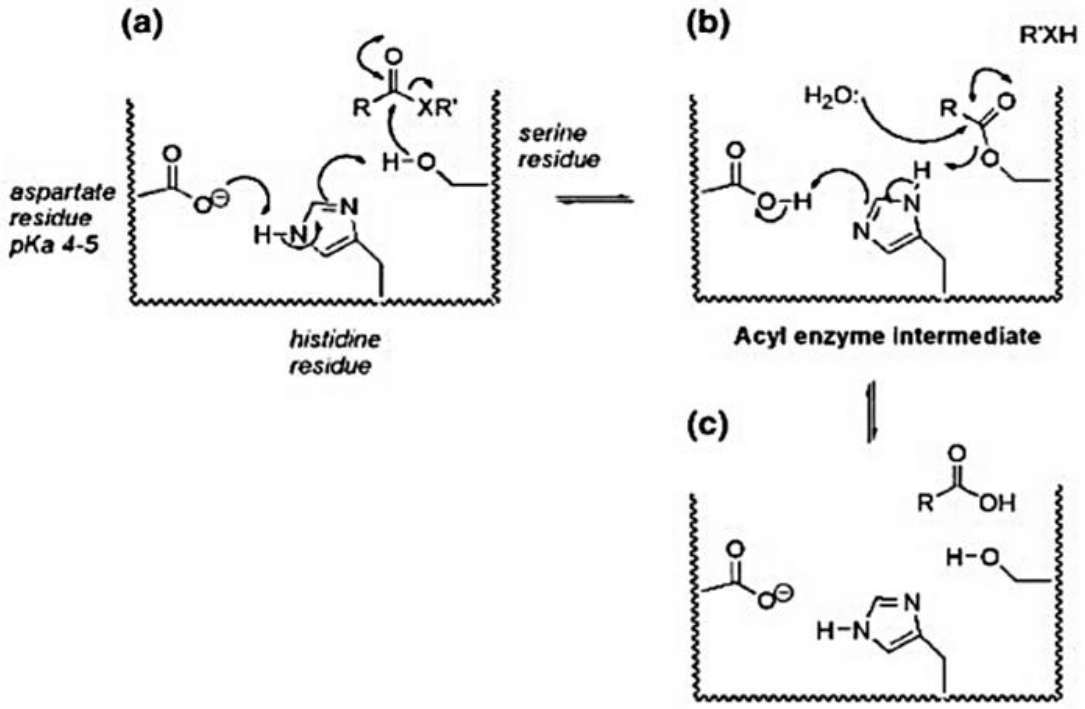



**Figure 3.** Mechanism of lipase catalysis (Reis *et al.*, 2009).

## 5. Optimization of enzyme production

In the process of producing enzymes using microorganisms, the steps that need to be considered include the selection of strains and regulation of process conditions.

Consideration of the use of microorganisms as a source of enzyme production, are:

1. Normally has a high specific activity per unit of product dry weight
2. There are no seasonal fluctuations, and there is no possibility of food shortages due to climate change
3. Broader characteristics of microorganism coverage, such as pH coverage, and temperature resistance
4. The genetic industry greatly increases rapidly, allowing optimization of yields and types of enzymes through strain selection, mutation, induction and selection of growth conditions

Production optimization needs to be done to maximize the value of protease enzyme production. Optimization with conventional methods takes a long time and is very expensive (Vohra and Satyanarayana, 2002). In using conventional methods in a single experiment, only one variable is varied so that one variable with another variable is not clearly known. Each variable is assumed to be independent of each other so it is necessary to do many tests in stages and there will be many variables studied during the production process (El Enshasy *et al*., 2008). The optimization process normally requires a long time with expensive costs (Saravanakumar *et al.*, 2010).

Several process conditions that must be optimized to gain the highest enzyme production are as follows:

- *Media composition*. The composition of the selected media must be sufficient to meet the nutritional needs that function as a food source for microorganisms.
- *Inducer*. Inducer is a substance that can stimulate the work of microorganisms to produce a particular enzyme. To produce lipase enzymes, inducers are usually used in connection with oils, fats, or fatty acids, such as palm oil.
- *pH and temperature*. The growth of microorganisms is influenced by pH, and usually the optimum pH of cell growth is different from the optimum pH of enzyme activity (Tambun, 2002). Enzymatic reactions are strongly influenced by temperature. Temperature can determine the maximum activity of an enzyme. The optimum temperature depends on the type of enzyme, the composition of the liquid, and the duration of the experiment.
- *Agitation and stirring*. Agitation functions to supply oxygen for cell growth, while stirring acts to make the fermentation mixture homogeneous.

## 6. Functional properties and safety aspects of starter culture

The principal considerations in the use of starter culture at traditional small-scale fermentation are whether this will make a significant contribution on: (i) improving processing conditions and product quality (more faster or accelerated fermentation processes); (ii) indicating more predictable of the fermentation process; (iii) generating more desirable of the sensory attributes; (iv) reducing the hygienic and toxicological risk (Holzapfel, 2002). Therefore, several functional properties and safety

attributes must be considered with and investigated first before applying the starter culture into the food fermentation process.

### 6.1 Antimicrobial activity

The antimicrobial activity against pathogenic bacteria or food-borne pathogenic is one important functional characteristics of starter cultures. Many lactic acid bacteria (LAB), staphylococci and enterococci, have been screened for the antagonistic activity towards several common pathogenic microorganisms. The production of metabolites such as diacetyl, acetaldehyde, hydrogen peroxide, organic acids (lactic and acetic), and bacteriocins, strongly affected the antagonistic activity of most starter cultures (Suskovic *et al*., 2010). Two main groups of antimicrobial substances were low molecular mass substances (<1000 Da) and high molecular mass (>1000 Da), including bacteriocins (Collins *et al*., 2009).

#### 6.1.1 Low molecular mass antimicrobials

The antimicrobial substances of LAB are deposited depend on the species, the environment, and the growth medium. The main low molecular mass metabolites are:

(i) *organic acids* – the antagonistic activity of this substances mostly because of the diffusion across cell membrane of the undissociated form of the molecule which subsequently hinders the crucial metabolic functions. The affected conditions including the fluctuations of cellular pH and membrane potential (Lorca and de Valdez, 2009).

(ii) *hydrogen peroxide* – the inhibitory effect of hydrogen peroxide is mainly caused by its powerful oxidizing impact to the cell which could lead protein denaturation (Lindgren and Dobrogosz, 1990).

(iii) *diacetyl, acetaldehide, and acetoin* – these compounds mostly cause the growth of contaminants, jointly with other antimicrobial substances (Vanderbergh, 1993).

(iv) *carbon dioxide* – $CO_2$ antimicrobial activity is attributed to its inhibition on enzymatic decarboxylations and to its influence on membrane permeability (Lorca and de Valdez, 2009).

(v) *reuterin and reutericyclin* – both metabolites inhibit Gram-positive bacteria, while the latter has a broader spectrum, including Gram-negative bacteria, fungi and protozoa (Kuleasan and Çakmakçi, 2002; Gänzle and Vogel, 2003).

(vi) *other low molecular mass antimicrobials* – including phenyllactic acid, hydroxy fatty acids, benzoic acid, methylhydantoin and mevalonolactone, which can inhibit Gram-positive and Gram-negative bacteria, moulds and yeasts (Suskovic *et al*., 2010).

### 6.1.2 High molecular mass antimicrobials

High molecular mass of antimicrobials mostly are proteinaceous substances with narrow-spectrum or broad-spectrum bactericidal activity, defined as bacteriocins. The bacteriocin can be divided into 3 or 4 classes: (i) class I including lantibiotics and lanthionine-containing single- and two-peptide which affect post-translational modification; (ii) class II, including non-lanthionine bacteriocins, which divided into class IIa (pediocins-like), class IIb (two-peptide bacteriocins), class IIc (circular bacteriocins), and class IId (lactococcin-like); and (iii) class III (bacteriolysins, lytic proteins, and often murein hydrolases). Some authors also proposed (iv) class IV bacteriocins that require other primary metabolites such as lipid and carbohydrate for their activity (Table 4).

**Table 4**. Classification and characteristics of bacteriocins

| Classification | Main characteristics | Examples |
|---|---|---|
| Class I | | |
| Lantibiotics or lanthionine-containing bacteriocins subdivided into: | small (<5 kDa) active peptides containing atypical amino acids: | |
| type A lantibiotics | – long peptides with positive charge | nisin, lactocin S |
| type B lantibiotics | – smaller peptide with negative or no net charge | mersacidin |
| Class II | | |
| non-lanthionine-bacteriocins: | small (10 kDa) and heat-stable post-translation unmodified non-lantibiotics | |
| subclass IIa | pediocin-like | pediocin PA1, sakacin A, leucocin A |
| subclass IIb | two-peptide | lactococcin G, lactacin F, plantaricin A |
| subclass IIc | circular bacteriocins | acidocin B, enterocin P, reuterin G |
| subclass IId | lactococcin-like | nukacin |
| Class III | | |
| Bacteriolysins | Large (>30 kDa) and heat-labile, with function to lyse the susceptible cells by catalyzing the hydrolysis of cell wall | enterolysin A, helveticin J, helveticin V-1829, lysostaphin |
| Class IV | | |
| Others | complex bacteriocins carrying lipid or carbohydrate moieties | lactocin 27, leuconocin S, pediocin SJ1, plantaricin S |

Source: Suskovic (2010).

Bacteriocins activity is generally targeted against low G+C Gram-positive bacteria. Several mechanism activities of bacteriocins have been revealed, such as inflection of enzyme activity, inhibition, or development of cell membrane pores.

Bacteriocins can associate with abundant anionic lipids present in the membranes, thus stimulate the pores development in membrane cells (Moll *et al*., 1999). However, the generalization of membrane disturbance patterns cannot sufficiently explain the mechanism activity of bacteriocins. Instead, particular objects appear participating in the pore development and other activities (Hechard and Sahl, 2002).

### 6.2 Hemolytic activity

Among the various determinants of pathogenicity possessed by a microorganism, hemolytic activity often acts as an initial factor for determining whether there is a pathogenicity of a microorganism in terms of laboratory identification. Hemolysis is the process of breaking down blood cell membranes caused by hemolysin protein. Hemolysis can carry out by two circumstances, intravascular or extravascular process. In the intravascular mechanism, the red blood cells (RBC) were destructed by complement lysis, which usually by immunoglobulin M (IgM), whereas in the second mechanism, the RBC were destructed by immune cells which recognized immunoglobulin G (IgG). The rupture of blood cell membrane causes the release of hemoglobin outside from red blood cells (Flegel, 2015; Rajkumar *et al*., 2016).

Diverse types of bacteria possessed several types of hemolytic proteins. These proteins act by integrating themselves with the RBC membrane or making holes at the membrane so that the structure of the membrane cell is disrupted. So far, there are three types of hemolytic proteins as follows: (i) β-hemolysin, shows digestion of lysis and a clear zone will appear around the colony; (ii) α-hemolysin, shows only partially lysing hemoglobin and green zone visible around the colony; (iii) γ-hemolysin (or non-hemolytic) refers to the absence of hemolytic activity (Oh and Jung, 2015).

### 6.3 Biofilm formation ability

Bacterial biofilm, as a sessile life form, ensures existence of bacterial life forms and it is a dominant phenotype in the nature over the free floating, planktonic form (Hallstoodley and Stoodley, 2005). Biofilm formation (Figure 4) is regulated by different genetic and environmental factors. The mechanism of biofilm formation mainly following four steps as follows: (i) attachment of the bacterial cells; (ii)

formation of the microcolony; (iii) maturation of the biofilm; and (iv) detachment. The inaugural step in forming biofilm relies upon the contact between the environment changes and the signaling regulation of bacteria. This attachment mainly affected by the properties of the surface to be attached. Following the attachment, the microcolony will be formed between the bacterial cell and others on the surface of, for example the humid food or the apparatus used in the food processing. Afterwards, maturation of the biofilm including its formation architecture, is regulated by several adhesive factors, such as accumulation-associated protein (Aap), eDNA, and polysaccharide intercellular adhesion (PIA). The dispersion of biofilm may occur if disruptive factors are added. The last stages which is detachment process can be carried out by the unstable environment, such as the reaction between the various enzymes with the exopolysaccharides (EPSs). This bacterial biofilm formation can be seen in Figure 4 (Zhao *et al*., 2017).

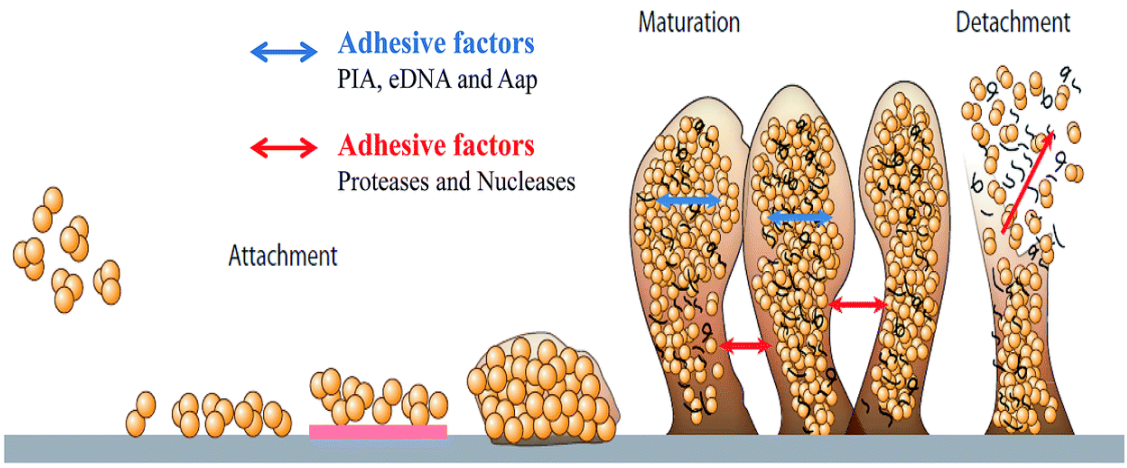



**Figure 4.** Biofilm formation (Zhao *et al*., 2017).

### 6.4 Antibiotic susceptibility

Conducting the antibiotic susceptibility test is crucial to validate a microorganism prior to be used in many applications related with human and animal purposes. Antibiotic susceptibility test also can give suitable results about antibiotics resistance of microorganisms so that it can be a good guidance for physicians or

researchers in the selection of the proper and effective microorganisms or the antibiotics to be applied (Atsbaha *et al*., 2017).

### 6.4.1 Development of antibiotic resistance in bacteria

The resistances of bacteria to antibiotics were probably developed ever since the clinical applications of antibiotics itself, when bacteria could adapt themselves and grow well even in the existence of the antibiotics (WHO, 2015). Two main factors, the existence of resistance genes and the improper use of antibiotics, were accountable for the development of antibiotic resistance in bacteria (Levy, 1998). Antibiotic resistance can be divided into two main types, intrinsic and acquired resistance. Intrinsic resistance could happen because of a certain inherent characteristic possessed by the bacteria. This intrinsic resistance is not harmful for non-pathogenic bacteria. On the other hand, acquired resistance might horizontally transferred among resistance bacteria to non-resistance bacteria. This acquired resistance could be exist through mutations or acquisition of genes encoding the bacterial resistance mechanism. This genetic change might modify the self-protective function of the bacteria become devastated. In addition, environmental changes, such as radiation effect and pH change, can also contribute to antibiotic resistances in bacteria (Poole, 2001; Davies and Davies, 2010).

The general mechanisms of bacterial resistances to antibiotics including target modification, efflux, immunity and bypass, and enzyme-catalyzed destruction (Figure 5). Target modification can occur through mutation of the targets themselves. Efflux can carry out by a group of protein pumps which expel the antibiotics outside from the cell. Immunity occurs when the antibiotics are bound by the proteins which subsequently cause the antibiotics cannot bind to its target. Enzyme-catalyzed destruction is recognized as the most specific mechanism in bacterial antibiotic resistance. In this mechanism, the enzyme recognized certain part of antibiotics and alter the interaction ability between the antibiotics and its target. For example, β-lactamase recognized and hydrolyzed the β-lactam ring of antibiotics so that the antibiotics cannot function properly (Wright, 2010).

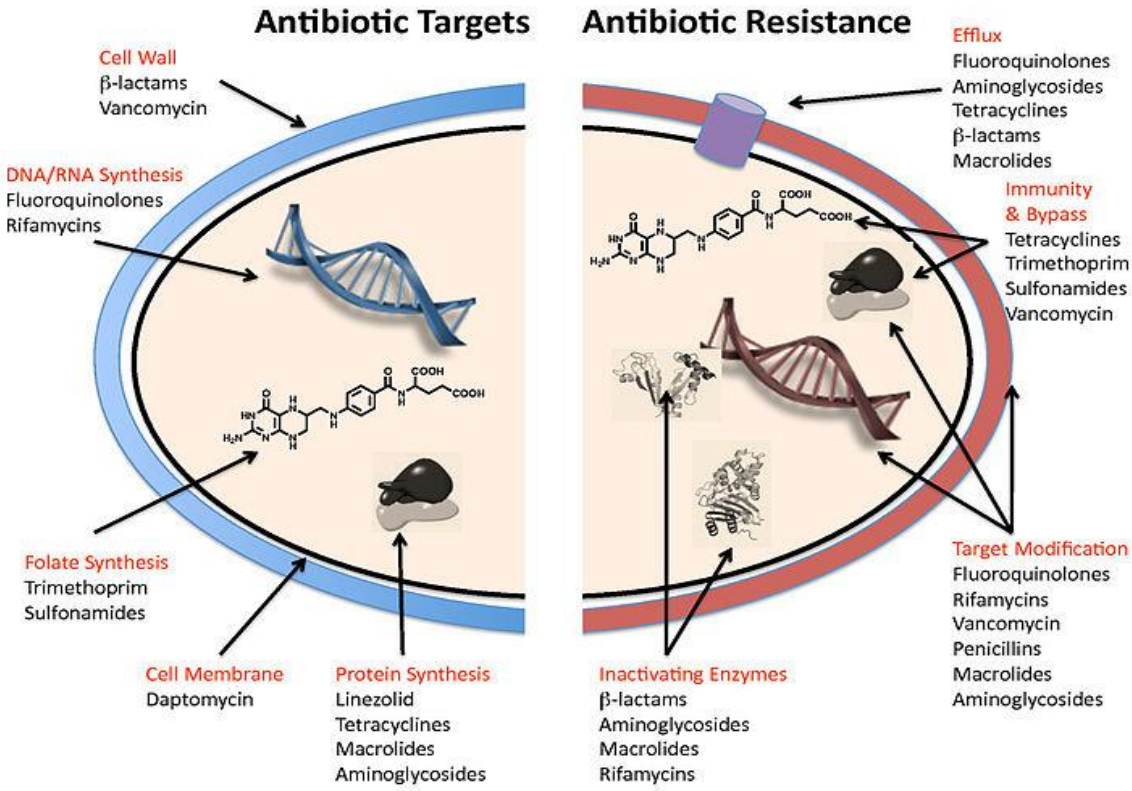



**Figure 5.** Targets of antibiotics and mechanism of antibiotic resistency (Wright, 2010).

### 6.4.2 Antibiotic resistance in bacteria isolated from foods

The World Health Organization (WHO) indicated that the world had reached a critical point due to the level of resistance of microorganisms to drugs used in treating several common infectious diseases (WHO, 2001). This antibacterial resistance may cause many advanced and life-threatening human infections. There has been a lot of literature that shows that many problems of infectious diseases throughout the world are caused by antimicrobial resistant bacteria and more virulent strains of pathogenic microbes that have evolved. Some clinically important microorganisms that have been able to develop resistance to available antimicrobials also include bacteria in foodborne infections (Levy and Marshall, 2004).

The commensal bacteria have been investigated by many researchers that can be a possible threat in antibiotic resistance. This is because those commensal bacteria can behave as a pool of similar antibiotic resistance genes in pathogenic bacteria discovered in human body. This is somehow particularly important in explaining of how antibiotic resistances are sustained and blowout throughout their populations (Levy and Miller, 1989). In addition, the pool of the antibiotic resistance bacteria can be discovered in many kinds of foods and beverages since they also contain other non-pathogenic bacteria. This will cause the transferring of antibiotic resistance genes of pathogenic bacteria to the non-pathogenic bacteria (Levy and Marshall, 2004). Therefore, antibiotic-resistance bacteria can distribute to the populations of humans and animals, through food consumption and the environment. This transmission is affected by migration of human and animal (WHO, 2015). A figure depicting how the bacterial antibiotics can spread from food and food environments can be seen in Figure 6.

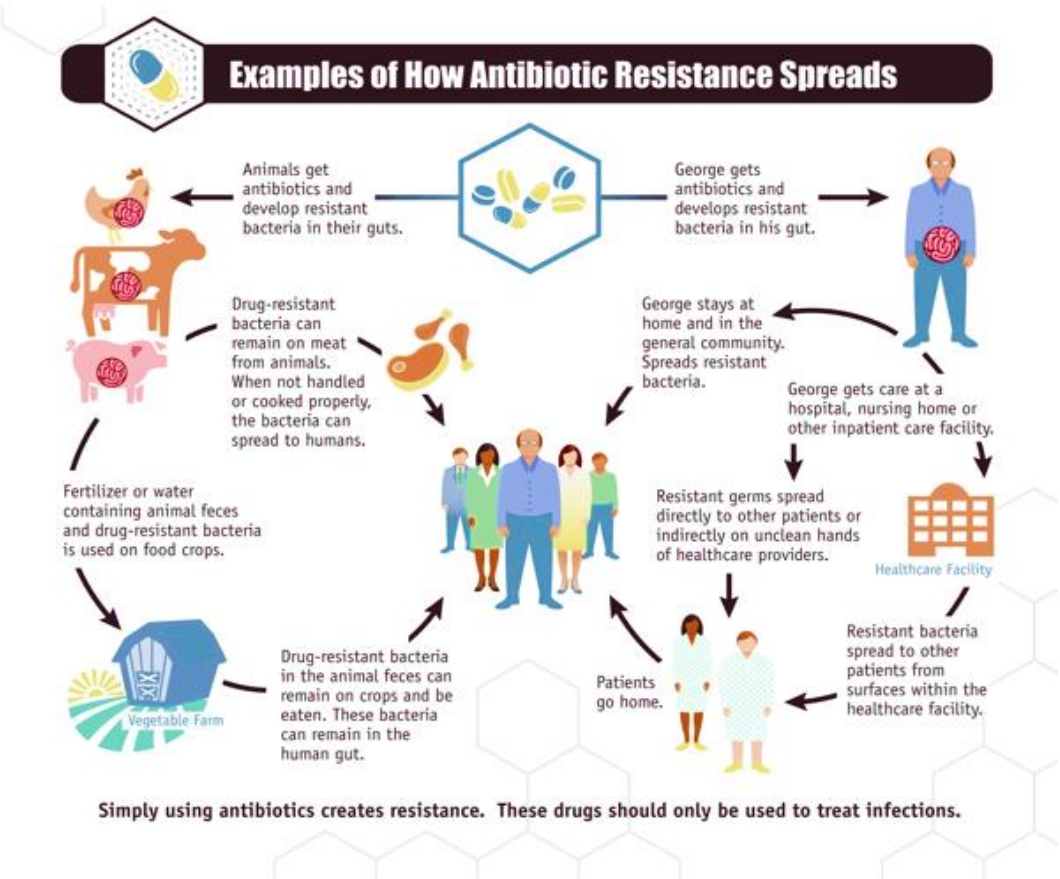



**Figure 6.** Examples of how antibiotic resistance spreads through food and environment from and to human and animal (CDC, 2013).

Temmerman *et al.* (2003) have investigated a total of 55 European probiotic products according to the identity and the resistency to antibiotics of bacteria isolated from the products. They have found that, among 236 isolates, 79% was detected showed resistency to kanamycin, 65% to vancomycin, 26% to tetracycline, 23% to penicillin G, 16% to erythromycin, and 11% to chloramphenicol. In addition, a total 68.4% of the isolates showed multiple resistance to antibiotics. Moreover, Charteris *et al*. (1998) obtained that bifidobacterial probiotics have shown resistance to colistin, fusidic acid, gentamycin, kanamycin, metronidazole, nalidixic acid, norfloxacin, polymyxin B, streptomycin, trimethoprim, and vancomycin, which is most likely to be intrinsic resistance, while susceptibility of these bifidobacterial proteins were recognized to ampicillin, bacitracin, cephalosporin, chloramphenicol, clindamycin, erythromycin, nitrofurantoin, penicillin G, and tetracycline. Several other cases of antibiotics resistant bacteria were also detected in the food processing environments such as in dust on farms (Chapin *et al*., 2005), in air streams inside and outside of feeding places of pigs (Sapkota *et al*., 2007; Brooks and McLaughlin, 2009), and soils under food crops, which fertilized manure containing antibiotics (Dolliver *et al*., 2008).

### 6.4.3 Global action plan on antimicrobial resistance

Antimicrobial resistance (AMR), a broader problem of antibiotic resistance, influences human health in all aspects and has severe impact on entire society. AMR also affects global economy by economic shortfalls because of the reduction of productivity by sickness and increased treatment cost (for both humans and animals). In line with this, WHO sets out the global action plan on AMR with five strategic objectives: (1) improving consciousness and understanding of AMR throughout effective training, education, and communication; (2) strengthening the knowledge throughout research and investigation; (3) reducing the number of infection through hygiene and sanitation environment; (4) optimizing the utilization of antibiotics for humans and animals health; (5) ensuring any sustainable investment for countering AMR. By applying this approach, the effort to ensure the quality-assured, safely, and

effectively treatment and prevention of various infectious diseases could be achieved (WHO, 2015).

Meanwhile, before the implementation of global action plan on AMR was announced by WHO, the European Union was established a qualified presumption safety (QPS), in which all microorganisms intended to be applied to animal or human must pass the safety assessment (Figure 7). In this assessment, all microorganisms intended to be applied in human or veterinary importance must be initially determined their susceptibility to antibiotics. An instrument for assessment of antibiotic susceptibility is by the determination of minimum inhibitory concentration (MIC) of the microorganism to the specified certain antibiotics. The MIC, which expressed as mg/L or μg/mL was applied so that susceptibility or resistance of bacteria can be determined. The level of susceptibility and resistance of a microorganism will be a comparison between the MIC of the microorganism determined and the cut-off value for each respective antibiotic. This cut-off value was determined by thorough research based on data clinic, therapeutic indication, dosage, pharmacokinetics, and pharmacodynamics, of the antibiotics (EFSA, 2014).

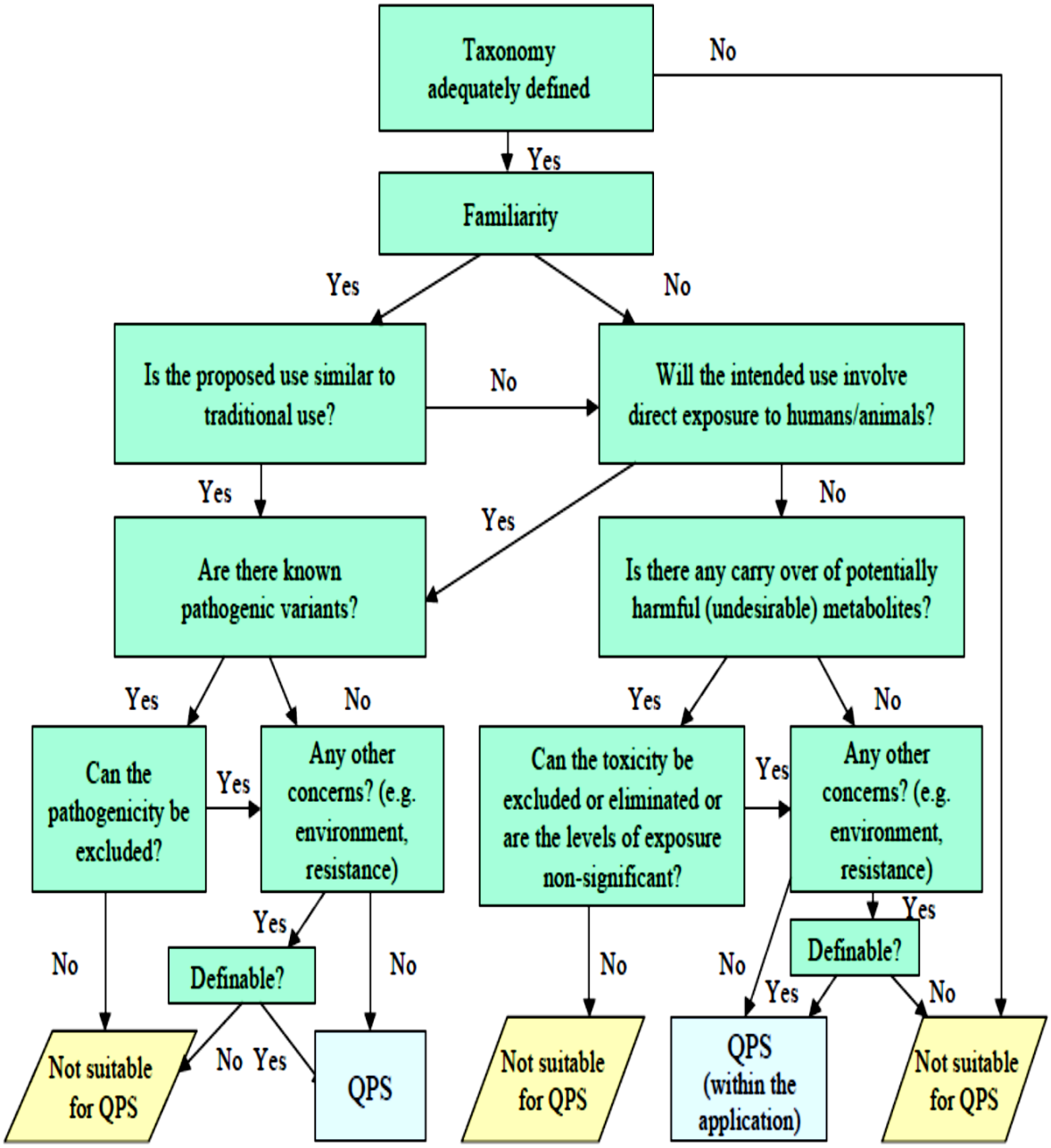



**Figure 7.** A general scheme for evaluating QPS status of microorganisms (EU, 2003).

### 6.5 Biogenic amines

Biogenic amines (BAs) are low molecular weight organic bases, which formed primarily by amino acids decarboxylation or by carbonyl compounds amination and transamination. There are two main types of the chemical structure of important BAs. The first group is aliphatic structure (putrescine, cadaverine, spermine, spermidine), and the second is aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine). Based on the number of amine groups, the BAs can be grouped into mono amines (tyramine and phenylethylamine), diamines (cadaverine, histamine, and putrescine) or poly amines (spermine and spermidine). These amines are synthesized through the metabolism of microorganisms, plants, and animals (Brink *et al*., 1990; Maijala *et al*., 1993).

A brief metabolic pathway of these BAs formation can be seen in Figure 8. Histidine decarboxylase converts histidine to histamine, while tyrosine decarboxylase converts tyrosine to tyramine. In addition, lysine decarboxylase converts lysine to cadaverine, while ornithine decarboxylase converts ornithine to putrescine. Putrescine can also be formed by agmatine deiminase system in which arginine firstly converted to agmatine and subsequently converted to putrescine. This polyamine metabolism pathway also enabling formation of bigger BAs, such as from putrescine to spermidine and by the sequential reaction of synthase, convert the spermidine to spermine (Halasz *et al*., 1994; Bover-Cid and Holzapfel, 1999; Medina *et al*., 2003). Of all BAs, histamine is the most significant amine and is thoroughly studied.

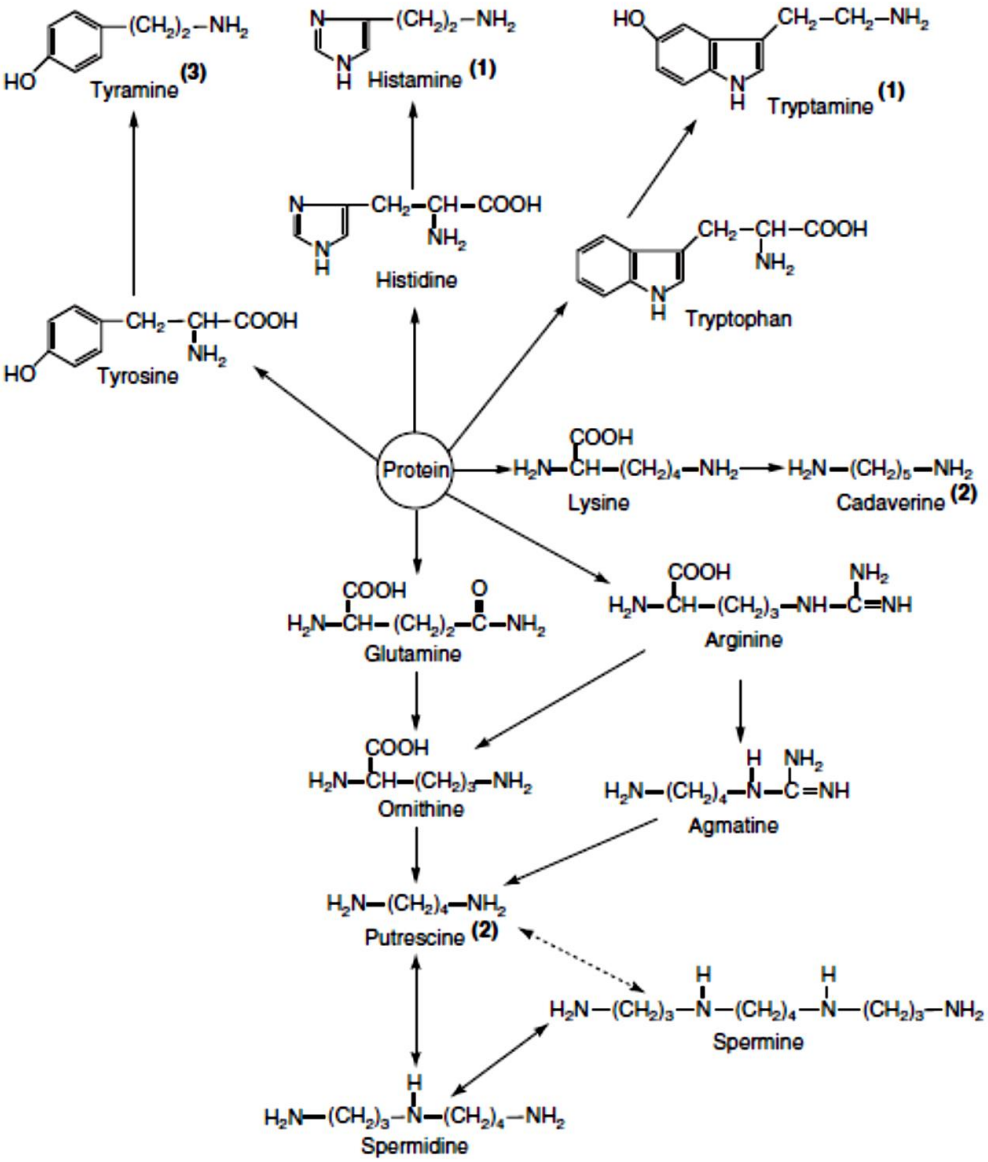



**Figure 8.** A brief metabolic pathway of several important biogenic amines formation in fish: (1) heterocyclic amine; (2) aliphatic amine; (3) aromatic amine (Halasz *et al*., 1994).

### 6.5.1 Factors affecting decarboxylase activity

BAs in food can be controlled through rigorous and comprehensive monitoring of good hygiene in the handling of raw material and manufacturing process so that inhibiting the growth of destructive microorganisms. In fermented food products, short fermentation times with application of active starter cultures compared

to spontaneous fermentation will help in preventing the formation of toxic amines. It is essentially to inhibit and prevent the decarboxylase activity of bacteria, since BAs are formed by enzymatic activity of food or bacteria. The important conditions considering formation of BAs in food by microorganisms are: the adequacy free amino acids, the suitable conditions for microbial growth, and the presence of microorganisms as the decarboxylase producers (Brink *et al.*, 1990; Marklinder and Lonner, 1992).

Temperature was the most factor to be considered in decarboxylase activity, since it is greatly affecting the formation of BAs in fish or seafood product. The highest amount of histamine production is the synchronization work of the production of histidine decarboxylation and the temperature optimum for histidine decarboxylase activity by the bacteria (Mendes, 2009). The production of histamine was insignificant above 50$^{o}$C and below 0$^{o}$C (Frank and Yoshinaga, 1984). The maximum activity of histidine decarboxylase was obtained when the bacteria was incubated around room temperature (25$^{o}$C), while for mesophilics attained at 37$^{o}$C and psychrophilics at 20$^{o}$C (Eittenmiller *et al*., 1981; Okuzumi *et al*., 1984). However, there is still lots of argued about the temperature optimum for the production of BAs by bacteria. Some studies have found optimum temperature for histamine production was around 15-20$^{o}$C (Yamanaka *et al*., 1984), while others reported around 30-38$^{o}$C (Frank *et al*., 1983; Pan, 1985).

### 6.5.2 Biogenic amines in fermented fish products

Generally, BAs can be likely to be produced from food or raw material involved in which the conditions allowing decarboxylase activity of the microorganisms. The origin and the microorganisms present are greatly affecting the number of BAs produced (Brink *et al.*, 1990).

BAs are widely exist in food and beverage products, such as fish, meat, dairy, and vegetable (Brink *et al.*, 1990). Several researches have been done to investigate the content of BAs in seafood product, and it has been found that the levels of the BAs can be varied greatly. Mizutani *et al.* (1992) could detect omithine, citrulline, and histamine in fish sauce. Yankah *et al* (1993) studied the changing of nitrogenous compounds throughout the processing of a Ghananian fermented fish. Some researchers

studied about the level of BAs in anchovies and tuna, and they obtained high level of BAs during the long manufacturing time which affect the bacterial growth responsible in producing BAs (Silla Santos, 1996; Veciana Nogue *et al.*, 1997). Auerswald *et al* (2006) have found high levels of histidine in Scombroid fish (*Thyrsites atun*) which in turn resulted the formation of histamine. Mohamed *et al*. (2009) investigated the BAs content in *Feseekh*, an Egyptian salted-fermented fish, and they found that the level of BAs was increased during processing and storaging. Meanwhile, Ezzat *et al* (2015), detected high level of histamine in Javanese fermented fish, *pekasam*.

### 6.5.3 Safety aspects and regulatory issues for biogenic amines production

BAs particularly influences various human's physiological functions. However, the toxic effect will arise if the high amount of BAs is consumed through the contaminated food. The ingesting of food containing high amount of BAs could potentially create histamine and tyramine poisoning, the two BAs possessed higher toxicity compared to others (Chong *et al*., 2011). The histamine poisoning is known as scromboid poisoning, because the symptoms are arised when people consumed spoiled fish from Scombridae or Scombresocidae. Type of fishes include are skip jack, bonito, mackerel, tuna, blue fish, and others (FDA, 2001). Other fishes often associated with scromboid poisoning also included anchovy, amberjack, herring, marlin, western Australian salmon, sockeye salmon, swordfish, and cape yellow tail (Price and Melvin, 1994). Indications of scombroid poisoning included itchy feelings around the mouth or throat, irritations around upper body, headaches, respiratory disorders, diarrhea, vomiting, nausea, and others. These symptoms initially occur within first few hours of digestion until 12 h to a few days. The symptoms will become severe if higher amount of poison was consumed, while individual sensitivity to histamine was also gave effect (Lehane and Olley, 2000). Meanwhile, the toxicological effect of tyramine might result in headache, nausea, migraine, vomiting, and hypertension (Ladero *et al*., 2010). Other BAs, such as cadaverine, putrescine, spermidine, and spermine might not have adversative health consequences, but occasionally they can trigger the formation of nitrosoamines, the carcinogenic amines compounds (Onal *et al*., 2013). They can also

trigger the toxicological effect of histamine by inhibiting the activity of diamine oxidase and N-methyltransferase, so that the histamine can be released to the intestinal fluid (Ibe *et al*., 1991). The oral toxicity level of tyramine, cadaverine, and putrescine, are above 2000 ppm, while spermine and spermidine are greater than 600 ppm (Til *et al*., 1997).

Currently, only histamine is already regulated by European Union (EU), USA, and several countries for its maximum limits contained in food product because it has the most toxicological effects for human being compared with other BAs. Table 5 shows some regulations already set by EU, USA, and other countries.

**Table 5.** Regulatory limits of histamine in seafood products

| Country/Institution | Limit | Reference |
|---|---|---|
| EU | 1. Fishery products from fish species associated with high amount of histidine, $n$ = 9, $c$ = 2, $m$ = 100 mg/kg, M = 200 mg/kg<br>2. Fishery products which have undergone maturation treatment in brine, $n$ = 9, $c$ = 2, $m$ = 200 mg/kg, M = 400 mg/kg | EC, 2005 |
| USFDA | 50 ppm (50 mg/kg) | FDA, 2011 |
| Australia, Germany, and New Zealand Food Standard Codes (FSC) | 200 mg/kg | Ezzat *et al*., 2015 |
| South Africa and Italy | 100 mg/kg | Ezzat *et al*., 2015 |

*n* number of units comprising the sample, *c* number of sample units giving values over *m* or between *m* and *M*

## 7. Application of starter cultures in seafood fermentation

Most of the traditional fermented products in developing countries are accounted as natural fermentations and are frequently produced in a non-controlled condition. Traditional seafood fermentation is a low-cost preservation method; moreover, it is often combined with the addition of salt or drying to reduce water activity and eliminate putrefying microorganisms in seafood (Beddows, 1997). The risk is that it is difficult to control the process and spoilage microorganisms can be incorporated in the process leading to unsafe products (Battcock and Azam-Ali, 1998). Nowadays, research done on fermented products is more into using starter cultures.

It has been proven that pure cultures isolated from a mixed population of microorganisms in traditional fermented foods show a variety of metabolic activities that vary greatly even among strains. The various metabolic activities include differences in growth rates, adaptation to certain substrates, ability to reduce antinutritionals, antimicrobial activities, attributes of taste and quality, as well as competitive growth characteristics in mixed cultures (Holzapfel, 1997). Therefore, single- and mixed-strain cultures to be used as starter cultures must be tested first on a pilot scale, before being used in small scale operations.

Caplice and Fitzgerald (1999) explained that introducing starter cultures is a good method to optimize the fermentation process. It improves the sensorial quality and microbial stability of fermented food products. It had been noted to be of use in important food groups like dairy products and fermented fruits, vegetables, meat, and fish. Several basic criteria of a good starter for fermented products are ability to improve the production process, enhance the sensorial characteristics of the final product and increase food safety (Holzapfel, 2002; Tamang *et al*., 2016). Starters are produced naturally from the original food product by keeping a sample from the previous fermentation process or in a ready-to-use, highly concentrated commercial form (Hansen, 2002). Though traditional producers of fermented products do not yet fully grasp the technology, Hwanhlem *et al.* (2011) mentioned that starter cultures have been used as a requirement to optimize small-scale fermentation processes in Thailand.

Application of starter culture to assist the seafood fermentation has been reported. As reported by Yongsawatdigul *et al.* (2007), three strains possess proteinase

activity, *Virgibacillus* sp. SK 33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5, have been applied as starter culture in the production of fish sauce. Udomsil *et al.* (2011) also reported the application of *Tetragenococcus halophilus* in the fish sauce fermentation. Meanwhile, Sarojnalini and Suchitra (2009) reported the application of *Bacillus* sp. and *Staphylococcus* sp. in the production of fermented fish, *Ngari*. Recently, Pongsetkul *et al*. (2018) reported the application of *Bacillus* sp. KC-3 as starter culture in Kapi production. All the reports showed that the application of starter cultures could result in the shorter time of fermentation process as well as the higher quality of product and the overall sensory characteristics.

**Scope of the study**

Fermented shrimp paste samples (Kapi) were collected from various traditional producers in Songkhla Province, Thailand. The best strains of bacteria isolated from shrimp paste samples exhibiting the highest activity of protease and lipase were selected and identified. Technological and safety attributes of the selected strains were also be investigated in order to obtain the safe strain. Strains exhibiting the safety aspect were then be applied as starter cultures for producing Kapi. The physicochemical characteristic and microbiological safety of the Kapi products were also investigated.

**Objectives of the study**

- To isolate, screen, and identify as well as characterize the best strain of protease- and lipase- producing bacteria from Kapi
- To study functional properties and safety aspects of the selected strains
- To study the optimum condition of protease and lipase production from the selected strains
- To apply the protease- and lipase-producing strains as starter culture for shortening the production time and improving the quality of Kapi

# CHAPTER 2

# *Virgibacillus halodenitrificans* MSK-10P, A POTENTIAL PROTEASE-PRODUCING STARTER CULTURE FOR FERMENTED SHRIMP PASTE (KAPI) PRODUCTION


## Abstract

Fermented shrimp paste (Kapi) is traditionally produced using a high content of salt and mainly consumed as a food condiment by the Thai people. In order to select potential functional autochthonous starter culture, the protease-producing bacteria from Kapi were isolated, screened, and evaluated for technological and safety aspects. Among 195 isolates, five bacterial strains (MSK-3P, MSK-4P, MSK-5P, MSK-7P, and MSK-10P) exhibited the highest activity of protease using Anson method. All of them were identified as *Virgibacillus halodenitrificans* using 16S rRNA sequence analysis. All selected strains exhibited growth in the presence of NaCl up to 25%. Neither strains showed hemolytic activity nor biogenic amine formation. Among all selected strains, only strain MSK-10P had no ability to form a biofilm, while four other strains showed a weak ability. Strain MSK-7P exhibited multiple resistances to tetracycline and vancomycin, while strains MSK-3P, MSK-4P, and MSK-10P showed susceptibility to all antibiotics tested. According to the obtained results, this protease-producing *V. halodenitrificans* MSK-10P is a good candidate for further investigation for use in Kapi fermentation to assess its technological performance as an autochthonous starter culture.


**Keywords:** protease-producing bacteria, Kapi, *Virgibacillus halodenitrificans*, starter culture, fermented shrimp paste

## 2.1 Introduction

Undoubtedly, fermentation plays a significant role in food industry today. Almost one-third of total food consumption worldwide is of fermented foods (Borresen *et al*., 2012). They are derived when group of indigenous microorganisms are presented naturally in raw materials or starter cultures to convert the substrates into palatable foods accepted by consumers (Holzapfel, 1997). Kapi is a Thai traditional fermented shrimp paste that is mainly consumed as a food condiment in many cuisines of the Thai people. It is fermented naturally by mixing tiny marine shrimp (mostly from *Acetes* sp.) and 10–30% (w/w) salt. The mixture then is dried under sunlight and fermented for several months (~ 1–3 months) (Faithong *et al*., 2010). Some recipes produce Kapi in less than 1 month, but these pastes are low-grade quality (Phithakpol, 1993). However, prolonging the fermentation time more than 5 months causes not only the decrease of amino nitrogen but also increases the level of ammonia that is harmful to human health (Prapasuwannakul and Suwannahong, 2015). As a result, variations in raw materials, amount of salt, fermentation conditions, and the length of production time affect the quality of Kapi. Therefore, the need for a method that will improve the quality of Kapi is indispensable.

Controlling fermentation by using a starter culture could be the best method to improve the quality of Kapi. Application of starter culture to improve the fermentation process in fishery products has been widely reported. Protease-producing bacteria such as *Virgibacillus* sp. SK 33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1–5 have been used in fish sauce production (Yongsawatdigul *et al*., 2007). The application of these starter cultures could significantly reduce fermentation time from 12 to only 4 months. The bacteria also increased the desirable volatile compounds as well as the free amino acids content in the fish sauce product. Similarly, the application of *Tetragenococcus halophilus*, isolated from fish sauce fermentation and having proteinase activity, could increase the level of desired amino acid content and the number of volatile compounds and reduce the content of biogenic amine (BA). Addition of this starter culture also shortened the fermentation time to 6 months (Udomsil *et al*., 2010).

Recently, *Bacillus* spp. K-C3 was applied as a starter culture in the production of Kapi (Pongsetkul *et al*., 2018). The starter was reported to accelerate the fermentation process and produce Kapi with favorable characteristics. By means of protease-producing bacteria, Kapi was produced within a shorter fermentation time, which is commercially advantageous for the industry. However, prior to implementation of starter culture in the fermentation process, a comprehensive investigation must be conducted to determine the technological and safety attributes. Accordingly, the present study deals with the isolation, screening, and identification of protease-producing bacteria from Kapi production process as well as assessment of their technological properties and safety of the chosen strain to be applied as an autochthonous starter culture for Kapi production.

## 2.2 Materials and Methods

### 2.2.1 Isolation, screening, and identification of protease-producing bacteria

#### *2.2.1.1 Isolation and screening*

Three Kapi samples were collected from various traditional producers in Songkhla Province, Thailand. All Kapi samples were prepared by the local producers using sea salt and tiny shrimp (*Acetes* sp.) with the ratio of 1:5 (Figure 9). The samples were taken from three different time intervals within the first week of fermentation: (i) day 1; (ii) day 3; and (iii) day 7 after salts added (Figure 1). Accordingly, the total number for isolation and screening of protease-producing bacteria was 27 samples.

Twenty-five grams of Kapi samples were mixed with 225 mL NaCl solution (10%, w/v). The samples were homogenized using a stomacher (Seward 400 circulator, England) at 230 rpm for 30 seconds. The homogenates were then serially decimal diluted with 10% (w/v) NaCl solution. The samples were primary screened by spreading onto three different media which containing 10% NaCl: (i) modified M73 (mM73) agar plates containing (%, w/v) yeast extract 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, KCl 0.5, $CaCl_2$ 0.02, agar 1.5, and supplemented with skim milk 1.0 for screening protease-producing bacteria (Norberg and von Hofsten, 1969); (ii) de Mann, Rogosa, and Sharp (MRS) (HiMedia, Mumbai, India) plates supplemented with (%, w/v) skim milk 1.0,

bromocresol purple 0.02, and agar 1.5 for screening lactic acid bacteria (LAB) (Udomsil *et al*., 2010); and (iii) nutrient broth (NB) (HiMedia, Mumbai, India) supplemented with (%, w/v) skim milk 1.0, and agar 1.5 for other protease-producing bacteria (Amoozegar *et al*., 2008). Bacterial colonies exhibiting a clear zone around the colony were randomly picked and streaked on new corresponding agar medium in order to obtain pure cultures.

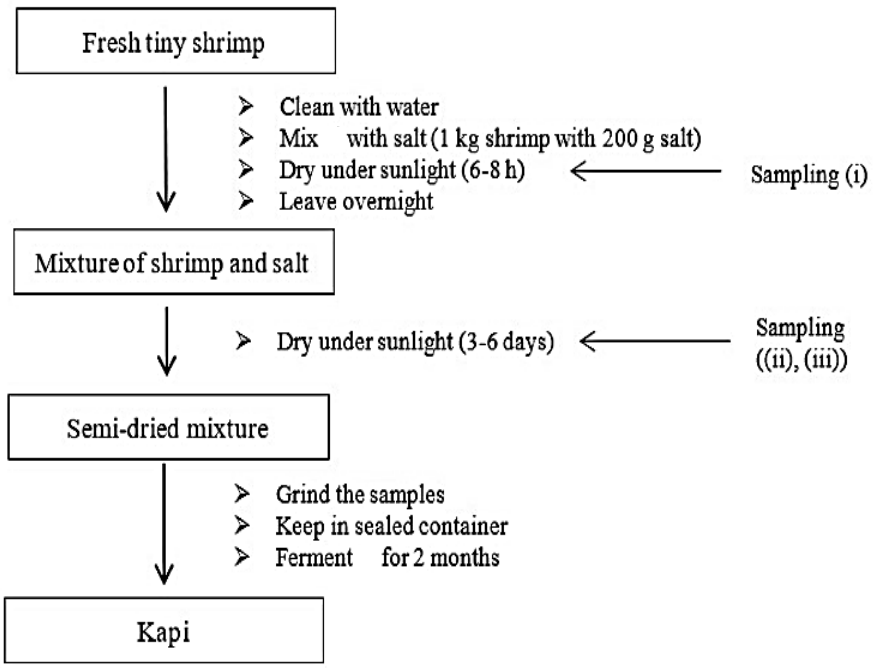



**Figure 9**. Scheme of Kapi production.

Secondary screening for selection of protease-producing strains was conducted using agar spot assay. Five microliters of overnight culture broth were dropped onto the corresponding agar medium (mM73, MRS, and NA) containing 1% (w/v) skim milk and then incubated at 30°C for 48 h. The relative enzyme activity (REA) was determined according to the formula as follow: REA = $(D^2 – d^2)/d^2$, where *D* is the diameter of clearing zone and *d* is the diameter of the colony (Bradner *et al*., 1999).

#### *2.2.1.2 Determination of protease activity*

The protease activity of selected strains (REA value > 1.0) was measured according to modified Anson method (Cupp-Enyard, 2008). The inoculum size of 1 % (approximately $10^6$ CFU/mL) was added into 10 mL of corresponding medium (mM73 or NB medium containing 1% w/v skim milk) and incubated under aerobic condition at 37ºC for 24h. The cell cultures were harvested by centrifugation at 9000 × *g* for 10 min. After that, 25 µL of culture supernatant was incubated with 130 µL of 0.6 % (w/v) casein in 50 mM sodium phosphate buffer (pH 8) at 37°C for 10 min. The reaction was terminated by adding 130 µL of 0.2 M trichloroacetic acid. Supernatant of the medium without any strains inoculations was used as a control. The reaction mixture was centrifuged, and the absorbance of soluble peptides in the supernatant were measured at 660 nm. One unit (U) of protease activity was defined as the amount of enzyme releasing 1 µmol of tyrosine equivalent per min. Isolates exhibiting the high activity of protease were selected for further investigation.

#### *2.2.1.3 Morphology and physiological properties*

The selected isolates were primarily tested by Gram staining, catalase production, and spore staining. The growth of the selected isolates at various temperatures (30-45ºC) was investigated in NB for 24 h. Various NaCl concentrations (0-30%, w/v) were also used to investigate the growth of the selected isolates in NB at 37°C for 24 h. After incubation, cell density was measured spectrophotometrically at OD 600 nm (Genesys-Thermo Science, Madison, USA).

#### *2.2.1.4 Molecular identification*

Genomic DNA of selected isolates was extracted using TIANamp Bacteria DNA kit (Tian Gen Biotech, Beijing, China) according to the manufacturer protocol and stored at -20ºC until use. The 16S rRNA region was amplified by polymerase chain reaction (PCR) with universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Turner *et al*., 1999). The PCR reaction was performed with following condition: initial denaturation at 95ºC for 5 min followed by 30 cycles of denaturation at 95ºC for

1 min, annealing at 55°C for 30 sec, elongation at 72°C for 30 sec, and final elongation step was performed at 72°C for 10 min. The generated amplicons were analyzed by agarose electrophoresis and observed under UV light. The PCR products were then sent to First Base Laboratories Sdn Bhd, Malaysia, for sequencing. Afterwards, the sequences were compared with known sequences using the basic local alignment search tool (BLAST program, http://www.ncbi.nlm.nih.gov/BLAST/). In addition, the 16S rRNA sequences of several type strains of Virgibacillus were downloaded from Gen Bank database in NCBI website (http://www.ncbi.nlm.nih.gov/). A phylogenetic tree was then built using the neighbor-joining method in the Molecular Evolutionary Genetics Analysis (MEGA) v7.0 package (Kumar *et al*., 2016).

### 2.2.2 Technological and safety aspects of selected protease-producing strains

#### *2.2.2.1 Antimicrobial activity*

Antimicrobial activity of selected strains was investigated by agar spot assay (Tulini *et al*., 2013). Briefly, 5 µL of overnight cultures were spotted onto NA plates. The plates were then incubated at 37°C for 24 h. Afterwards, each plate was overlaid with pathogenic indicator strains (*Bacillus cereus* DMST5540, *Escherichia coli* DMST4212, *Listeria monocytogenes* DMST17303, *Salmonella* Typhimurium DMST5784, *Staphylococcus aureus* DMST8840, and *Vibrio parahemolyticus* DMST5665) that were seeded in brain heart infusion (BHI) (HiMedia, Mumbai, India) soft agar (0.75%, w/v) medium. All plates were subsequently incubated at 37°C for 24-48 h. The antibacterial activity was determined by measuring the clear zone (radius in mm) around the colony which was indicated as growth-free inhibition zone.

#### *2.2.2.2 Hemolytic activity*

Blood hemolysis was investigated by growing the selected isolates in NB at 37°C for 18 h and then one loopful of each bacterial strain was streaked onto tryptone soya agar (TSA) plates (HiMedia, Mumbai, India) containing 5% (v/v) defibrinated human blood (group O) kindly obtained from Songklanagarind Hospital, Hat Yai, Thailand. All plates were incubated at 37°C for 24-48 h. Hemolytic reaction

was investigated as follows: β-hemolysis (shown as a clear zone around the colony), α-hemolysis (shown as a partial hydrolysis around the colony), or γ-hemolysis (if no clear zone around the colony). The negative hemolytic activity was shown by the γ-hemolysis (Oh and Jung, 2015). *S. aureus* DMST8840, which possesses β-hemolysis activity was used as a positive strain.

#### *2.2.2.3 Biofilm formation*

Biofilm formation ability was investigated in 96-well microtiter plates (Stepanovic *et al*., 2007) with slight modification. The microtiter plates were filled with 200 µL of bacterial suspension in tryptone soya broth (TSB) (HiMedia, Mumbai, India) supplemented with 1% (w/v) glucose and incubated at 37ºC for 24 h. The content of each well was then aspirated and washed three times with 300 µL of sterile phosphate buffer saline pH 7.2. The attached bacteria were fixed with 2% (w/v) sodium acetate for 15 min and then the plates were emptied and dried. Afterwards, each well was stained with 200 µL of 0.1% (w/v) crystal violet for 5 min. Excess stain was washed with deionized water and then the plates were air-dried. The dye bound to biofilm was resolubilized with 160 µL of glacial acetic acid. The optical density of each well was measured at 595 nm using a microplate reader (BioTek Instruments, Winooski, USA). Biofilm formation was interpreted as follows: N, non-biofilm forming ($A_{595} \leq OD_c$); W, weak ($OD_c < A_{595} \leq 2 \times OD_c$); M, moderate ($2 \times OD_c < A_{595} \leq OD_c$); S, strong ($A_{595} \geq 4 \times OD_c$). $OD_c$ was defined as the optical density cut-off value. Each sample was assayed in triplicates.

#### *2.2.2.4 Antibiotic susceptibility*

Mueller-Hinton broth (MHB, HiMedia, Mumbai, India) and antibiotic diluted in MHB (AMHB) were used for determining antibiotic susceptibility of selected strains. The broth microdilution method (Klare *et al*., 2005) was used to evaluate the Minimum Inhibitory Concentration (MIC) for each antibiotic. Briefly, a flat-bottom 96-well polystyrene microtiter plates was inoculated with 100 µL of AMHB containing serial antibiotics in several concentration ranges (µg $mL^{-1}$): ampicillin (0.063-64); chloramphenicol (0.125-256); clindamycin (0.02-32); erythromycin (0.063-64);

kanamycin (0.13-256); penicillin G (0.063-64); streptomycin (0.25-512); tetracycline (0.063-64) and vancomycin (0.13-256). Subsequently, each well was filled with 100 µL of selected isolates cultivated in MHB (final concentration was $10^4$ CFU $mL^{-1}$). Plates were then incubated at 37°C for 24 h. Bacteria inoculated in MHB were used as positive control and a bacteria-free well was used as negative control. After 24 h incubation at 37°C, a growth inhibition of selected strains was determined at OD600 using a microplate reader (Biotek Instruments). The inhibition was expressed in percentage by the equation: inhibition (%): [($OD_{600}$ of the positive control group – $OD_{600}$ of the group)/$OD_{600}$ of the positive control group] x 100. The $MIC_{90}$ was defined as the lowest concentration of antibiotics that inhibits bacterial growth at an inhibition rate of higher than 90% (Almeida *et al*., 2008). Susceptibility of the strains were defined based on the corresponding cut-off values for *Bacillus* sp. (EFSA, 2012). Consequently, strains showing MIC value higher than the corresponding cut-off value were determined as resistant.

#### *2.2.2.5 Biogenic amines production*

BAs production of selected strains was analyzed by thin-layer chromatography (TLC) (Garcia-Moruno *et al*., 2005). Selected strains were cultivated overnight in NB and then inoculated into 10 mL NB supplemented with 0.5% (w/v) of conforming amino acids precursor (Merck (Germany) and Sigma-Aldrich (Singapore): L-histidine monohydrochloride, L-tyrosine disodium salt, L-lysine monohydrochloride and L-ornithine hydrochloride. The cultivation was performed at 30°C for 7 days, and then cells were removed by centrifugation at 9000 × *g* for 10 min. Supernatant containing the corresponding BAs were converted into their fluorescent dansyl derivatives (Landeta *et al*., 2007) and subsequently spotted onto TLC plate (silica gel 60 F254, Merck, Germany). The separation of dansylated compounds were conducted using solvent combination of chloroform:di-ethyl ether:tri-ethylamine (3:2:1, v/v/v). The TLC plate was visualized using a Gel Doc™ EZ (Biorad, London, UK) system under UV light exposure. The authentic BAs stock solutions (histamine, cadaverine, putrescine, and tyrosine) (Sigma-Aldrich, Singapore) were made by preparing a 2% (w/v) solution in 40% ethanol (5% in the case of histamine).

The existence of genes encoding decarboxylase enzymes involved in BAs production was determined by PCR method using primers as shown in Table 6. PCR program was conducted with initial denaturing at 95°C for 5 min, followed by 36 cycles at 95°C for 1 min, at 53°C for 1 min, and at 72°C for 3 min with final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis gel agarose and observed under UV light.

**Table 6.** Primers used for the detection of genes encoding decarboxylase enzymes involved in biogenic amines production

| Gene[a] | Primer | Sequence[b] | Amplicon size (bp)[c] | Result of MSK-10P from PCR method | Reference |
|---|---|---|---|---|---|
| *hdc* | HIS1-F | GGNATNGTNWSNTAYGAYMGNGCNGA | 372 | - | De las Rivas |
| | HIS1-R | ATNGCDATNGCNSWCCANACNCCRTA | | | *et al*., 2006 |
| *tdc* | TDC-F | TGGYTNGTNCCNCARACNAARCAYTA | 825 | - | De las Rivas |
| | TDC-R | ACRTARTCNACCATRTTRAARTCNGG | | | *et al*., 2006 |
| *odc* | PUT1-F | TWYMAYGCNGAYAARACNTAYTTYGT | 1440 | - | De las Rivas |
| | PUT1-R | ACRCANAGNACNCCNGGNGGRTANGG | | | *et al*., 2006 |
| *ldc* | CAD2-R | CAYRTNCCNGGNCAYAA | 1185 | - | De las Rivas |
| | CAD2-F | GGDATNCCNGGNGGRTA | | | *et al*., 2006 |

[a]*hdc*, histidine decarboxylase; *tdc*, tyrosine decarboxylase; *odc*, ornithine decarboxylase; *ldc*, lysine decarboxylase.

[b] Y = C or T; R = A or G; W = A or T; S = C or G; M = A or C; D = A, G, or T; H = A, C, or T; B = C, G, or T; N = A, C, G, or T.

[c] Amplicon size is DNA fragment of gene encoding decarboxylase which have been amplified from pathogens.

### 2.2.3 Statistical analysis

All the experiments were performed in triplicate and the level of significance was ±5%. Analysis of Variance (ANOVA) and mean comparison by Duncan's Multiple Range Test (DMRT) were performed to compare the protease activities. In addition, correlation between REA and protease activity was statistically

analyzed using bivariate Pearson Correlation. A multivariate analysis with Principal Component Analysis (PCA) approach was performed to select the best strain for starter culture. All the statistical analysis was performed by using Statistical Package for the Social Science (SPSS 16.0 for Windows, SPSS Inc, Chicago, IL USA).

## 2.3 Results and Discussions

### 2.3.1 Isolation, screening, and identification of protease-producing bacteria

Isolation and screening of protease-producing bacteria is an important step in the selection of appropriate starter culture for the production of fermented seafood. *Halobacterium* sp. SP1, *Virgibacillus* sp. SK37, *Virgibacillus* sp. SK33, and *Staphylococcus* sp. SK1-9, have been demonstrated to accelerate the fermentation time of fish sauce production as well as improving the nutritional content of the fish sauce (Akolkar *et al*., 2010; Yongsawatdigul *et al*., 2007). In the present study, protease-producing bacteria were isolated and screened from the early stage of Kapi fermentation using agar plate medium containing skim milk for the first preliminary screening. Protease-producing microorganisms facilitated the degradation of the fish or shrimp tissues at the early stage of fermentation before subsequent hydrolyzing them into smaller parts at the later stage of fermentation (Fan *et al*., 2017). Meanwhile, total viable count and proteolytic bacterial count were higher in early days of fermentation of *Budu*, a Malaysian fish-sauce (Sim *et al*., 2015). In addition, the proteolytic and amylolytic bacterial count was also found to be higher in the early stage of fermentation than final stage fermentation of *Ronto,* an Indonesian fermented shrimp product (Khairina *et al*., 2016). Accordingly, it can be assumed that more protease-producing microorganisms can be obtained in early days of fermentation compared with at the final stage of fermentation.

It was found that a total of 195 isolates exhibiting clear zone around colony which were developed only in mM73 agar (79%) and NA (21%). None of the protease-producing LAB was obtained from MRS agar containing skim milk. This was probably because most protease-producing LAB could not grow at high salt concentration, and an aerobic condition within the first days of the Kapi production

process which consisted of abundant normal flora under the aerobic condition as well. Consequently, this could inhibit the growth of LAB (Kleekayai *et al*., 2015).

Among the obtained isolates, 12 isolates (~6%) exhibiting the considerable high REA value were chosen to be examined further for their protease activity (Table 7) in order to select the best protease-producing strain. Although REA value is expected to correspond with the protease activity (Bradner *et al*., 1999), sometimes it does not accurately reflect the actual amount of enzyme. This might be caused by the different microclimate conditions and growth environments between solid and liquid medium that would affect the production of enzymes (Jeanson *et al*., 2015). Some bacterial strains isolated from freshwater fish showed high protease in agar plate assay but did not show quantitatively high protease activity (Bairagi *et al*., 2002). In addition, the largest clear zone indicated high REA which showed protease activity did not correlate with high quantitative protease activity of bacteria in the gut of tiger shrimp (*Penaeus monodon*) (Bhowmik *et al*., 2015). However, as depicted in Figure 10, there was strong correlation between REA value and quantitative enzyme activity ($r>0.5$) and it was a statistically significant difference as well ($p<0.05$). In the present study, strains exhibiting high value of REA also had high protease activity. Therefore, five isolates (MSK-3P, MSK-4P, MSK-5P, MSK-7P, and MSK-10P) exhibiting high REA and high protease activity (>5.75 U/mL) (Table 7) were selected for further investigation.

**Table 7**. Comparison of protease activity from REA and Anson method of selected isolates

| Source | Isolate code | Medium | Relative enzyme activity (REA) | Anson method (U/mL) |
|---|---|---|---|---|
| A | MSK-1P | mM73 | $2.11\pm0.26^{ef}$ | $5.25\pm0.22^{e}$ |
| A | MSK-2P | mM73 | $2.82\pm0.28^{de}$ | $6.05\pm0.63^{d}$ |
| A | MSK-3P | mM73 | $5.68\pm1.20^{a}$ | $6.60\pm0.30^{c}$ |
| A | MSK-4P | NA | $4.64\pm1.27^{ab}$ | $5.85\pm0.08^{d}$ |
| A | MSK-5P | NA | $4.45\pm0.34^{bc}$ | $6.10\pm0.03^{d}$ |
| B | MSK-6P | mM73 | $3.39\pm0.30^{cd}$ | $0.50\pm0.01^{h}$ |
| B | MSK-7P | mM73 | $3.94\pm0.14^{bcd}$ | $8.94\pm0.11^{b}$ |
| B | MSK-8P | NA | $1.33\pm0.22^{f}$ | $2.59\pm0.05^{f}$ |
| C | MSK-9P | NA | $3.39\pm0.30^{cd}$ | $5.70\pm0.27^{d}$ |
| C | MSK-10P | NA | $4.45\pm0.13^{bc}$ | $9.47\pm0.09^{a}$ |
| C | MSK-11P | mM73 | $1.91\pm0.50^{ef}$ | $2.29\pm0.22^{f}$ |
| C | MSK-12P | mM73 | $1.33\pm0.08^{f}$ | $0.90\pm0.03^{g}$ |

A, producer A; B, producer B; C, producer C. Values are given as mean ± SD from triplicate determination. Different lowercase superscript letters in the same column indicate the significant differences at $p<0.05$.

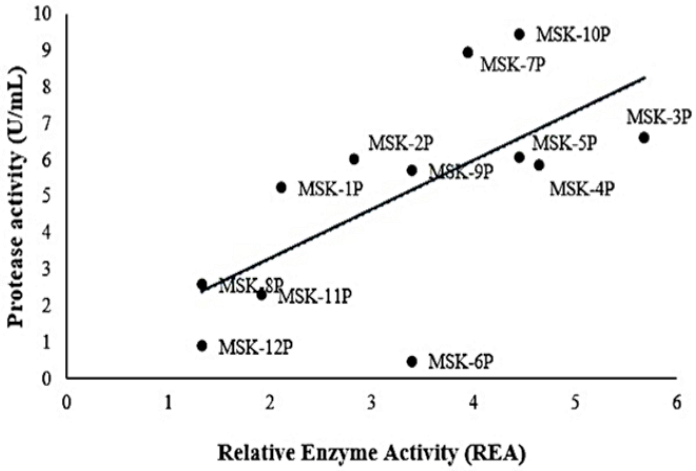



**Figure 10**. Pearson correlation graph between REA and protease activity determination of isolated protease-producing bacteria (*r* indicates the Pearson correlation; *p* indicates significance level; • represents the data pair of REA and protease activity; — indicates the correlation line).

### 2.3.2 Morphology and physiological properties

Morphological characterization studies revealed that all of five isolates were catalase positive, Gram-positive, and spore-forming bacteria (Table 8). In addition, all isolates were able to grow well at 30-40$^{o}$C and showed optimum at 35-37$^{o}$C, while they showed poor grow at 45$^{o}$C. Meanwhile, all isolates grew slightly in the absence of salt and at the salt concentration of 25-30%, and the optimum growth was shown at 10-20% NaCl. In general, the temperature range of bacterial growth is an important characteristic in the physiological and taxonomy studies of bacteria. Increased temperature will increase nutritional requirements for bacterial development (Chandler and McMeekin, 1989; Xu *et al*., 2018). Based on the physiological growth conditions of these isolated strains, it can be concluded that all strains belong to the mesophilic and halotolerant groups, because they have optimum growth conditions at 35-37$^{o}$C and salt concentrations of 10-20%. This condition causes these strains to survive under temperature around 35-37$^{o}$C during the Kapi production. Moreover, salt tolerance of all isolates indicated the potential use of them as the starter candidates in Kapi production since it contained about 10-30% (data not shown).

**Table 8.** Morphology properties of selected protease-producing bacteria

| Characteristics | Isolates | | | | |
|---|---|---|---|---|---|
| | MSK-3P | MSK-4P | MSK-5P | MSK-7P | MSK-10P |
| Gram staining | + | + | + | + | + |
| Catalase test | + | + | + | + | + |
| Spore staining | + | + | + | + | + |
| Growth at different temperature ($^{o}$C)[a] | | | | | |
| 30 | ++ | ++ | ++ | ++ | ++ |
| 35 | +++ | +++ | +++ | +++ | +++ |
| 37 | +++ | +++ | +++ | +++ | +++ |
| 40 | ++ | ++ | ++ | ++ | ++ |
| 45 | + | + | + | + | + |
| Growth at different NaCl concentrations (%, w/v)[a] | | | | | |
| 0 | + | + | + | + | + |
| 5 | ++ | ++ | ++ | ++ | ++ |
| 10 | +++ | +++ | +++ | +++ | +++ |
| 15 | +++ | +++ | +++ | +++ | +++ |
| 20 | +++ | +++ | +++ | +++ | +++ |
| 25 | + | + | + | + | + |
| 30 | + | + | + | + | + |
| 35 | - | - | - | - | - |

[a]The bacterial growth at different temperatures and NaCl concentration was indicated as follows: (+++) means fully (OD600 >2); (++) means moderately (OD600 ~ 1–2); (+) means poorly (OD600 ~0.1–1); (-) means no growth (OD600 <0.1).

### 2.3.3 Molecular identification

Five selected protease-producing strains were identified by 16S rRNA sequencing analysis. It was found that all five strains belong to the species *Virgibacillus halodenitrificans* with 98-99% similarity (data not shown). This was supported by the phylogenetic tree analysis as shown in Figure 3. All the sequences were deposited in GenBank under accession numbers MG241245, MG241246, MG241247, MG241248,

and MG241249 for isolates MSK-3P, MSK-4P, MSK-5P, MSK-7P, and MSK-10P, respectively.

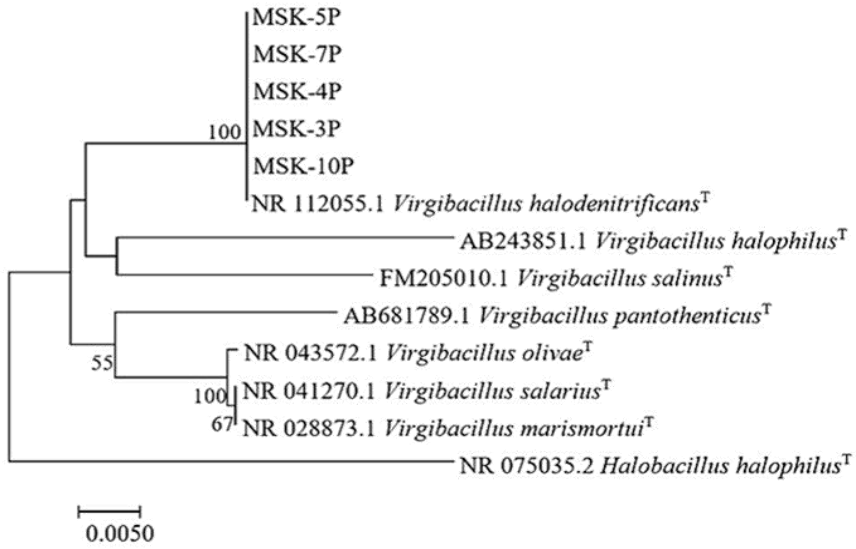



**Figure 11.** Phylogenetic tree of the type strain of *Virgibacillus* species and selected protease-producing bacteria isolated from Kapi. The tree is constructed based on the results of 16S rRNA sequencing. The scale bar corresponds to five nucleotide substitutions per 1000 nucleotides (evolutionary distances). Numerals indicate the statistical significance of the order of branching, determined by "bootstrap" analysis of 100 alternative trees; values less than 50% are not shown.

Generally, *Virgibacillus* is a Gram-positive, spore-forming, catalase positive, and halotolerant bacterium, which is widely found in many habitats, mostly isolated from saline environments (Chen *et al*., 2009; Wang *et al*., 2010; Xu *et al*., 2018). This bacterium can also be found in some food products, for instance fish sauce (Yongsawatdigul *et al*., 2007); chicken meat (Gupta *et al*., 2008); *jeotgal*, a traditional Korean fermented seafood (Kim *et al*., 2011); and dairy products (Seiler and Wenning, 2013). In addition, a draft of genome sequence of *V. halodenitrificans* 1806 has been reported (Lee *et al*., 2012). The authors indicated that *V. halodenitrificans* 1806 contains several genes contributing to the osmolarity for uptaking compatible solutes essential in hypersaline conditions which responsible for salt-tolerance properties.

### 2.3.4 Technological and safety aspects of selected protease-producing strains

#### *2.3.4.1 Antimicrobial activity*

The ability of the selected strain to prevent the growth of pathogens is one important aspect in the selection of starter culture. The antibacterial activity against pathogens will make the fermented food safe for consumption (Ratanaburee *et al*., 2013). However, in the present study, all isolated virgibacilli strains did not exhibit the antibacterial activity toward any indicator pathogenic strains (*Staph. aureus* DMST 8840, *E. coli* DMST 4212, *Sal.* Typhimurium DMST 5784, *B. cereus* DMST5540, *V. parahemolyticus*, and *L. monocytogenes* DMST 17303). Even though the selected protease-producing strains did not exhibit antimicrobial activity toward indicator pathogenic strains, Kapi might be safe from those pathogens because it contained high salt content. Although some food-borne pathogens demonstrated moderate salt tolerance, almost all pathogens showed low-salt tolerance. *Staph. aureus* can survive up to 15-20% NaCl, but its toxin can only be produced at 5-10% NaCl (Taormina, 2010). Death of *E. coli* occurs when cells are suspended in 8.5% NaCl media (Glass *et al*., 1992), meanwhile the growth of *L. monocytogenes* is inhibited by the addition of 11% NaCl (Zarei *et al*., 2012). Exposure to 5% NaCl showed decrease in cells of *B. cereus* (den Besten *et al*., 2006), while *Sal*. Typhimurium can only adapt until 0.5% NaCl (Thayer *et al*., 1987). Generally, Kapi is produced in high salt concentration (10-30% w/w NaCl); therefore, certain pathogens cannot grow in this harsh condition. Indeed, salt is necessary not only in complex food formulation and recipes but also in the prevention of foodborne microbial pathogens (Taormina, 2010).

#### *2.3.4.2 Hemolytic activity*

Hemolytic activity has been linked to virulence in many pathogenic microorganisms and can be a risk to human health. Therefore, strains exhibiting β-hemolytic activity may not be applicable as starter candidates. In the present study, hemolytic activity of selected protease-producing strains was also investigated. All selected protease-producing strains displayed γ-hemolytic activity (Table 9). This means that all strains did not induce hemolysis (Oh and Jung, 2015) and may be safe to

be used as the starter culture. However, other virulence factors should be further investigated before use as autochthonous starter culture.

**Table 9.** Hemolytic activity and biofilm formation ability of selected protease-producing bacteria

| Isolate code | Hemolytic activity[a] | Biofilm formation ability[b] |
|---|---|---|
| MSK-3P | - (γ) | W |
| MSK-4P | - (γ) | W |
| MSK-5P | - (γ) | W |
| MSK-7P | - (γ) | W |
| MSK-10P | - (γ) | N |
| *Staph. aureus* DMST4212 | + (β) | M |

[a] + (β) means the isolate had β-hemolytic activity; - (γ) means the isolate had γ-hemolytic activity

[b] M means moderate activity, W means weak ability, N means no activity

#### *2.3.4.3 Biofilm formation ability*

Bacterial biofilm can be classified as one of a specific cause in bacterial pathogenicity in the human body. Based on recent studies, biofilm formation emerged as a potential key feature associated with virulence (Coenye *et al*., 2007; Fox *et al*., 2005). According to the definition of biofilm formation ability (Stepanovic *et al*., 2007), it was found that four strains (MSK-3P, MSK-4P, MSK-5P, and MSK-7P) showed weak ability of biofilm formation. Only one strain (MSK-10P) did not exhibit the ability of biofilm formation (Table 9).

#### *2.3.4.4 Antibiotic susceptibility*

Antibiotic resistance is one of the major safety issues for starter culture selection, because starter culture can be a potential contributing vector of antibiotic to microbiota through the fermentation process (Podkowik *et al*., 2016). These determinants can spread to humans through food consumption (EFSA, 2012). In this

study, antibiotic susceptibility of all selected protease-producing strains was tested using nine antibiotics (ampicillin, chloramphenicol, clindamycin, erythromycin, kanamycin, penicillin G, streptomycin, tetracycline, and vancomycin). It was found that isolates MSK-3P, MSK-4P, and MSK-10P showed sensitivity to all antibiotics tested. Strain MSK-5P showed resistance to tetracycline and strain MSK-7P resisted to both tetracycline and vancomycin (Table 10). However, vancomycin resistance was not a concerning principle factor for application of a microorganism in food and feed (EFSA, 2012). Different results were reported by Santos *et al*. (2010) who found that *Virgibacillus* sp. H31 isolated from Brazilian's sponge-associated bacteria showed resistance to penicillin G and chloramphenicol but was sensitive to erythromycin and tetracycline.

**Table 10.** Antibiotic susceptibility of selected protease-producing bacteria

| Antibiotics[a] | Cut-off value ($\mu g\ mL^{-1}$)[b] | $MIC_{90}$ of isolate | | | | |
|---|---|---|---|---|---|---|
| | | MSK-3P | MSK-4P | MSK-5P | MSK-7P | MSK-10P |
| Amp | n.r.[c] | 0.5 | 0.25 | 0.5 | 4 | 0.26 |
| Cam | 8 | 8 | 4 | 4 | 4 | 4 |
| Clin | 4 | 2 | 0.13 | 0.5 | 0.15 | 1 |
| Ery | 4 | 0.5 | 0.13 | 0.13 | 0.26 | 0.26 |
| Kan | 8 | 0.26 | 0.5 | 0.5 | 1 | 0.26 |
| Pen G | n.r.[c] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Str | 8 | 4 | 2 | 8 | 4 | 4 |
| Tet | 8 | 2 | 0.25 | 16[d] | 16[d] | 2 |
| Van | 4 | 2 | 2 | 0.5 | 16[d] | 4 |

[a]Amp, ampicillin; Cam, chloramphenicol; Cli, clindamycin; Ery, erythromycin; Kan, kanamycin; Pen G, penicillin G; Str, streptomycin; Tet, tetracycline; and Van, vancomycin

[b]The cut-off values as suggested by EFSA (2012)

[c]n.r.: not required

[d]showed resistance to corresponding antibiotics

The selection of the best starter culture should be performed by using multivariate analysis, considering there are five strains that have their own technological and safety attributes. PCA was chosen to select the best strain for starter culture, since this approach is a beneficial tool for selecting and discriminating strains with various positive characteristics that are clearly differentiated from other strains (Bevilacqua *et al*., 2012; Rodriguez-Gomez *et al*., 2012). By means of PCA as depicted in Figure 12, *V. halodenitrificans* MSK10-P exhibited the best characteristics for a starter culture. The strain MSK-10P showed positive correlation to all five variables tested (BF, AST, PAM, REA, and HA). However, strains MSK-3P and MSK-4P showed positive correlations only to variables BF, AST, and REA, strain MSK-7P only to PAM and HA, and strain MSK-5P showed negative correlations to all variables tested. Therefore, strain *V. halodenitrificans* MSK10-P was selected as the best strain for starter culture and further subjected to investigate whether it produced BA.

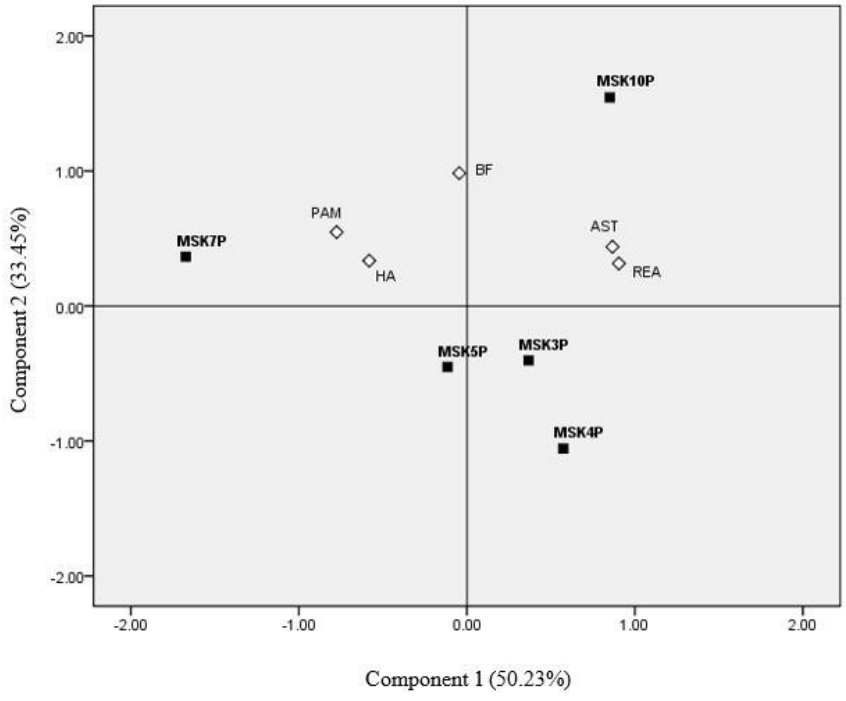



**Figure 12**. Principal component analysis (PCA) for the selection of the best strain as starter culture in the Kapi production production (◇ variables used for PCA; ■ strains; PAM: protease activity-Anson method, HA: hemolytic activity; BF: biofilm formation; AST: antibiotic susceptibility test; REA: relative enzyme activity).

#### *2.3.4.5 Biogenic amine production*

BAs are basic organic nitrogen compounds formed by decarboxylation of amino acids (Spano *et al*., 2010). BAs can be the clue to food poisoning (Talon and Leroy, 2011). BAs obtained in fermented food products are intensely stimulated by composition and microbial growth during handling process and storage (Carelli *et al*., 2007). Some of the most important BAs found in seafood are histamine, putrescine, cadaverine, agmatine, tryptamine, tyramine, spermine, and spermidine (Jeya Shakila, *et al*., 2001). Two important BAs, histamine and tyramine are considering as antinutritional complexes; meanwhile, putrescine and cadaverine somehow do not have strong unhealthy effect. However, those two latter BAs can expand the toxicological effect of histamine by blocking its metabolizing enzymes (Hungerford and Arefyev, 1992, Veciana-Nogués, *et al*., 1997). The toxicity of these four BAs has led to the general agreement that they should not be allowed to accumulate in food (Spano *et al*., 2010). A good starter culture should not affect the food product by producing the BAs (Talon and Leroy, 2011). Therefore, the ability of a starter culture candidate in producing BAs should be assessed.

In the present study, the ability of *V. halodenitricans* MSK-10P to produce BAs was investigated using both phenotypic and genotypic analysis. It was found that *V. halodenitrificans* MSK-10P was considered as a non-BAs producer as determined by TLC method (Figure 13) and confirmed by PCR method (Table 1). As summarized in Table 1, no amplicons of related gene encoding amino acid decarboxylases were detected by *V. halodenitrificans* MSK-10P using standard primers. Accordingly, these results demonstrate that *V. halodenitrificans* MSK-10P is safe as a possible alternative candidate strain for application as a starter culture in the Kapi production.

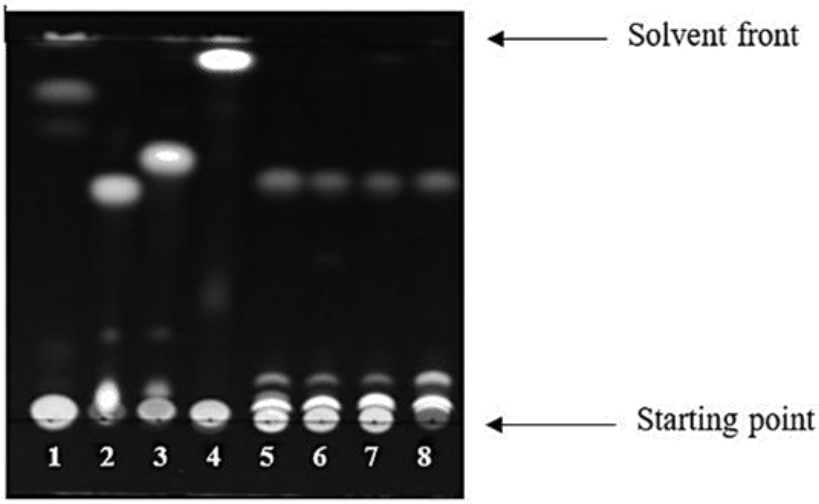



**Figure 13**. Dansylated biogenic amines standards as separated by TLC: histamine (1), putrescine (2), cadaverine (3), and tyramine (4). Cell-free supernatants of *V. halodenitrificans* MSK-10P grown in NB medium containing substrate: histidine (5), ornithine (6), lysine (7), tyrosine (8).

## 2.4 Conclusions

On the basis of all results obtained, protease-producing *V. halodenitrificans* MSK-10P, isolated from Kapi exhibited high protease activity as well as tolerance to high salt environment. This study provided comprehensive information about the safety attributes of this strain including negative activity in hemolysis, biofilm production, and BAs production. This strain also exhibited susceptibility to all antibiotics tested. The majority of tested virulence factors were also absent, confirming the safety. All these features are important factors that should be considered in the selection of good starter culture. Therefore, *V. halodenitrificans* MSK-10P could be a promising starter culture for Kapi production. Application of this strain might accelerate the fermentation process and increase the quality of nutritional content of Kapi as well.

# CHAPTER 3

# SELECTION AND EVALUATION OF TECHNOLOGICAL AND SAFETY CHARACTERISTICS OF AUTOCHTONOUS LIPASE-PRODUCING BACTERIA FROM FERMENTED SHRIMP PASTE (KAPI)


## Abstract

Lipase-producing bacteria have the function for producing specific aroma and improving the taste of fermented food. The aims of this study were to isolate, screen, and characterize the technological properties as well as safety aspects of lipase-producing bacteria isolated from Kapi, to be a potential autochthonous starter culture for Kapi production. Four bacterial strains (LSM3, LSM4, LSM15, and LSM16) exhibiting the highest lipase activity based on olive oil-rhodamine B agar plate and copper-soap method were selected. All of them were identified as *Staphylococcus* sp. using 16S rRNA sequence analysis and specific primers for housekeeping genes of staphylococci. All the strains exhibited growth in the medium containing NaCl up to 25%. In addition, strains LSM3, LSM15, and LSM16 showed non-hemolysis on the blood agar plate however, displayed weak ability in biofilm formation. Strains LSM4, LSM15, and LSM16 exhibited antagonistic activity toward *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*. The antibacterial activity against only Gram-positive bacteria came from bacteriocin-like inhibitory substance (BLIS). Strains LSM15 and LSM16 showed susceptibility to all antibiotics tested. Furthermore, neither strains had classical staphylococcal enterotoxin (SE) encoding genes nor gene encoded decarboxylase enzymes. Based on all results, LSM16 could be selected and applied as a promising starter culture in the production of Kapi.


Keywords: lipase-producing bacteria, Kapi, *Staphylococcus* sp., safety attributes, starter culture

## 3.1 Introduction

Kapi is a Thai traditional fermented shrimp paste which is mainly consumed as a food condiment by Thai people. Kapi has gained considerable attention because it has been shown to have not only some beneficial-health properties, such as antioxidant activity, antifibrinolytic, and antihypertensive, but also has high content of fat, amino acids, and protein (Kleekayai *et al.*, 2015; Pongsetkul *et al.*, 2015).

Improving the quality of Kapi is an important attention. Since Kapi is generally made using traditional methods, accordingly the quality of Kapi become vary observed. One of the attempts can be used to improve the quality of Kapi is applying starter cultures in the fermentation process. Application of starter cultures is believed to improve food safety and to provide typical sensory quality of food. The best starter cultures mostly originated from autochthonous microflora as they are estimably adapted well with the condition throughout the fermentation process (Rebecchi *et al.*, 1998; Babic *et al*., 2011). In addition, these autochthonous starters are believed can dominate other microbiota in the process because they have specific metabolic abilities (Leroy *et al.*, 2006).

Lipolysis is believed to be a significant factor that influences the process of aroma formation. Hence lipolytic bacteria are needed in the fermentation process. Lipase-producing bacteria have been widely reported in the fermentation of dairy product, meat, sausage, and other derivative products of meat. Lipolytic staphylococci, in particular *Staph. xylosus*, *Staph. carnosus*, and *Staph. equorum* were proven their effects on the meat fermentation process by releasing free fatty acids for the aroma and flavor development (Søndergaard and Stahnke, 2002; Olesen *et al*., 2004). However, very few reports are available for lipase-producing bacteria isolated from fermented seafood especially from fermented shrimp paste (Kapi). Recently, Gao *et al*. (2017) have isolated and applied lipolytic-producing *Staph. xylosus* 135 as a starter culture in traditional low-salt fermented fish (Suan yu). This lipolytic starter culture increased the volatile flavor compounds in Suan yu. Daroonpunt *et al*. (2018) isolated several lipolytic bacterial strains from Thai fermented fish. They were identified as *B. amyloliquefaciens, Corynebacterium variabile, Staph. saprophyticus, Virgibacillus dokdonensis*, and *V. halodenitrificans*. Those strains were only characterized for their

lipase activity. However, their technological and safety attributes have not been investigated yet.

In this study, the lipase-producing bacteria were isolated, screened, and characterized for their important technological properties as well as safety attributes in order to discover the new potential autochthonous functional starter culture for Kapi production.

## 3.2 Materials and Methods

### 3.2.1 Isolation, screening, and identification of lipase-producing bacteria

#### *3.2.1.1 Sampling and isolation*

Kapi samples were prepared and sampled as previously described in Chapter 2. In order to isolate the lipase-producing bacteria, the diluted samples were spread onto three different media: (i) modified M73 (mM73) agar containing (g/L) yeast extract 10.0, $MgSO_4 \cdot 7H_2O$ 10.0, KCl 5.0, $CaCl_2$ 0.2, agar 15, gum arabic 1.0, NaCl 100 (Norberg and Hofsten, 1969), supplemented with olive oil 10.0; (ii) deMann, Rogosa, and Sharp (MRS) (HiMedia, Mumbai, India) agar supplemented with (g/L) bromocresol purple 0.2, agar 15, NaCl 100, olive oil 10.0, and gum arabic 1.0; and (iii) nutrient broth (NB) (HiMedia, Mumbai, India) supplemented with (g/L) agar 15, NaCl 100, olive oil 10.0, and gum arabic 1.0. All the media were blended by mixer using high speed 30 sec before autoclaving. Each dilution was analyzed in duplicate. The plates were then incubated at 30$^{o}$C for 3-5 days. Bacterial colonies exhibiting orangish zone around their colony were observed under UV light were randomly picked and streaked on new corresponding agar medium. The selected isolates were subcultured twice overnight in Tryptone Soya Broth (TSB, HiMedia, India) and were subjected to Gram staining, spore staining, and catalase production.

#### *3.2.1.2 Determination of lipolytic activities*

The activity of lipase produced by selected isolates were determined according to copper-soap method (Lee and Rhee, 1993). The inoculum size of 1% of the selected isolates (approximately $10^6$ CFU/mL) was added into 10 mL of corresponding medium (mM73 or NB medium containing 1% w/v olive oil) and shaking under 150 rpm at 37$^{o}$C for 24 h. The cell cultures were harvested by

centrifugation at 9000 × *g* for 10 min. After that, the supernatant as crude lipase exract (100 µL), 400 µL of 50 mM sodium phosphate buffer (pH 7.0), and 1 mL of emulsion containing 5% (w/v) olive oil in isooctane were incubated at 37°C with vigorously shaking at 200 rpm for 30 min. The enzyme reaction was terminated by adding 400 µL of 6 M HCl. Subsequently, 500 µL of upper isooctane layer containing the fatty acids was drawn off then added with 500 µL isooctane and 500 µL copper acetate solution. After mixing by vortex for 15 sec, free fatty acids dissolved in isooctane were determined by measuring the absorbance at 715 nm. One unit (U) of lipase activity was defined as the amount of enzyme released 1 µmol of oleic acid equivalent per min. The amount of free fatty acids was calculated based on the standard curve of oleic acid. All isolates exhibiting the high activity of lipase were selected for further experiment.

#### *3.2.1.3 Morphology and physiology properties*

The morphology of selected isolates was primarily tested by Gram staining, catalase production, and spore staining. Growth of selected isolates at various temperature was investigated by culturing 1% v/v inoculum in 10 mL of trypticase soya broth (TSB, HiMedia, Mumbay, India) at 30, 35, 37, 40 and 45°C for 24 h. In addition, growth in the presence of NaCl (0–30%, w/v) was also examined in TSB at 37°C for 24 h. After incubation under given condition, optical density at 600 nm ($OD_{600}$) was measured by spectrophotometer (Genesys, Thermo Science, USA).

#### *3.2.1.4 PCR analysis*

Molecular identification by 16S rRNA sequence analysis of selected lipase-producing bacteria were conducted with the same procedure as previously described in Chapter 2. Confirmation of identity gene for genus *Staphylococcus* were performed using the specific primers Tstag765 and TstaG422 which were designed by Martineau *et al.* (2001) as shown in Table 11. PCR was performed in total volume 25 µL consisted of 0.2 µM (each) of the primers, 1× DreamTaq mastermix, DNase-free water, and template staphylococcal DNA. Thermal cycling steps were as follows: initial denaturation at 94$^{o}$C for 3 min followed by 40 cycles of denaturation at 95$^{o}$C for 1 min,

annealing at 55°C for 30 s, and elongation at 72°C for 30; final elongation step at 72°C for 3 min.

**Table 11.** Primers used in this study

| Gene[a] | Primer | Sequence (5'-3')[b] | Predicted amplicon size | Result of amplification from LSM16[c] | Reference |
|---|---|---|---|---|---|
| | TstaG422 | GGCCGTGTTGAACGTGGTCAAATCA | 370 | + | Martineau *et al.*, 2001 |
| | Tstag765 | TIACCATTTCAGTACCTTCTGGTAA | | | |
| *icaA* | ica4F | TCTCTTGCAGGAGCAATCAA | 371 | - | Gad *et al.*, 2009 |
| | ica2R | TCAGGCACTAACATCCAGCA | | | |
| *nukA* | N174F | ATGGAAAATTCTAAAGTTATGAA | 174 | - | Hong *et al.*, 2014 |
| | N174R | TTATGAACAACAAGTAAATACAAA | | | |
| *nukM* | N185F2 | GGTGTAACGGAGTTACAGGAC | 185 | - | Hong *et al.*, 2014 |
| | N185R2 | TCCCATGACGCCATGACATAG | | | |
| *sea* | SEA_F | CCTTTGGAAACGGTTAAAACG | 126 | - | Moura *et al.*, 2012 |
| | SEA-R | CCTTTGGAAACGGTTAAAACG | | | |
| *seb* | SEB_F | GGTACTCTATAAGTGCCTGCTTC | 475 | - | Moura *et al.*, 2012 |
| | SEB-R | GCATCAAACTGACAAACG | | | |
| *sec* | SEC_F | AGAACTAGACATAAAAGCTAGG | 267 | - | Moura *et al.*, 2012 |
| | SEC-R | TCAAAATCGGATTAACATTATCC | | | |
| *sed* | SED_F | TTTGGTAATATCTCCTTTAAACG | 309 | - | Moura *et al.*, 2012 |
| | SED-R | CTATATCTTATAGGGTAAACATC | | | |
| *see* | SEE_F | CCTATAGATAAAGTTAAAACAAGC | 173 | - | Moura *et al.*, 2012 |
| | SEE-R | TAACTTACCGTGGACCCTTC | | | |
| *hdc* | HIS1-F | GGNATNGTNWSNTAYGAYMGNGCNGA | 372 | - | De las Rivas *et al*., 2006 |
| | HIS1-R | ATNGCDATNGCNSWCCANACNCCRTA | | | |
| *tdc* | TDC-F | TGGYTNGTNCCNCARACNAARCAYTA | 825 | - | De las Rivas *et al*., 2006 |
| | TDC-R | ACRTARTCNACCATRTTRAARTCNGG | | | |
| *odc* | PUT1-F | TWYMAYGCNGAYAARACNTAYTTYGT | 1440 | - | De las Rivas *et al*., 2006 |
| | PUT1-R | ACRCANAGNACNCCNGGNGGRTANGG | | | |
| *ldc* | CAD2-R | CAYRTNCCNGGNCAYAA | 1185 | - | De las Rivas *et al*., 2006 |
| | CAD2-F | GGDATNCCNGGNGGRTA | | | |

[a] *ica*A, intracellular adhesion A; *nukA*, nukacin ISK-1 structural gene; *nukM*, post-translational modification encoding gene for nukacin; *sea*, Staphylococcus enterotoxin A; *seb*, Staphylococcus enterotoxin B; *sec*, Staphylococcus enterotoxin C; *sed*, Staphylococcus enterotoxin D; *see*, Staphylococcus enterotoxin E; *hdc*, histidine decarboxylase; *tdc*, tyrosine decarboxylase; *odc*, ornithine decarboxylase; *ldc*, lysine decarboxylase.

[b] Y = C or T; R = A or G; W = A or T; S = C or G; M = A or C; D = A, G, or T; H = A, C, or T; B = C, G, or T; N = A, C, G, or T.

[c] (+) showed band correspond with the DNA fragment of targeting gene; (-) no band showed correspond with the DNA fragment of targeting gene.

### 3.2.2 Technological properties and safety assessment of selected lipase-producing isolates

#### *3.2.2.1 Proteolytic activity*

Determination of proteolytic activity of selected isolates was performed on TSA plates contained 10% (w/v) NaCl and supplemented with either 1% (w/v) skim milk or 1% (w/v) casein. All plates were incubated at 37ºC for 3-5 days. Clear zone around the colony was indicated as the proteolytic activity of the isolates (Thongthai and Suntinanalert, 1991).

#### *3.2.2.2 Antimicrobial activity*

Antimicrobial activity of selected strains was investigated by agar spot assay (Tulini *et al.*, 2013). Briefly, 5 μL of 24h-old cultures were spotted onto TSA plates. The plates were incubated at 37°C for 24 h. Each plate was then overlaid with approximately $10^6$ CFU/mL of pathogenic indicator strains (*B. cereus* DMST5540, *E. coli* DMST4212, *L. monocytogenes* DMST17303, *Sal.* Typhimurium DMST5784, *Staph. aureus* DMST8840, and *V. parahemolyticus* DMST5665) seeded in brain heart infusion (BHI, HiMedia, Mumbai, India) soft agar medium (0.75%, w/v). Afterwards, all plates were incubated at 37 ℃ for 24-48 h. The antimicrobial activity was determined by measuring the clear zone (radius in mm) around the colony which was indicated as growth-free inhibition zone.

Strains exhibiting the antimicrobial activity from previous method were subsequently investigated for their antibacterial compounds and inhibitory spectrum. Firstly, the strains were cultivated in 100 mL of BHI broth (BHI, HiMedia, Mumbai, India) at 37ºC for 18 h. Culture supernatant was collected by centrifugation (9000 × *g* for 30 min, at 4ºC). The supernatants obtained were treated as follows: (i) the cell-free supernatant (CFS) was used directly; (ii) the CFS was neutralized to pH 6.5 by using 6N NaOH to rule out the effect of organic acids (CFS-N); (iii) the CFS-N were treated with proteases (α-chymotrypsin, pepsin, proteinase K and trypsin; Sigma-Aldrich, Steinheim, Germany) with the final concentration of the protease was 1 mg/mL, and incubated at 37ºC for 3 h (CFS-NP). All the samples were filter-sterilized first (0.22 μm) before investigated. Antibacterial activity spectrum of the strains was determined

using the agar well-diffusion assay (Aslim *et al*., 2005). Twenty microliters of *Staph. aureus* DMST8840 ($10^6$ CFU/mL) which was used as indicator strain seeded in brain heart infusion (BHI, HiMedia, Mumbay, India) soft agar medium (0.75%, w/v) was then poured into sterilized petri dish. After that, 50 μL of each samples were dispensed into each well and incubated at 37ºC for 24 h. The result reported as the diameter of inhibition zone.

Molecular method was also applied to investigate the gene encoding antimicrobial peptides of the selected strains. The sequences of primer pairs were listed in Table 11. PCR mixtures contained the primers and 1 μg of genomic DNA. The thermal cycling conditions were 5 min at 95ºC, followed by 30 cycles of 30 s at 95ºC, 30 s at 46 or 61ºC, and 90 s at 72ºC, and a final incubation for 7 min at 72ºC. After PCR amplification, 10 μL of each reaction mixture was resolved in a 1.0% (w/v) agarose gel (Hong *et al*., 2014).

#### *3.2.2.3 Hemolytic activity*

Blood hemolysis was investigated by streaking the selected isolates in onto tryptone soya agar (TSA) plates containing 5% (v/v) defibrinated human blood (group O) at 37°C for 18 h, following the procedure as previously described in Chapter 2, section 2.2.2.2.

#### *3.2.2.4 Biofilm formation*

Biofilm formation ability was firstly investigated using the microplate titer method as previously described in Chapter 2. Detection of *ica*A gene by PCR was conducted as molecular technique to confirm and determine the ability of isolated strains for producing biofilm. The specific pair of primers (Table 11) and the PCR conditions to amplify *icaA* gene were performed using the method described by Møretrø *et al.* (2003). The thermal cycling conditions were: 5 min at 95ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 50ºC and 1.5 min at 72ºC. The amplified products were resolved on 1.5% agarose gel.

#### *3.2.2.5 Antibiotic susceptibility*

The antibiotic susceptibility test was performed using the same method as previously described in Chapter 2, except the concentration ranges (μg/mL) of antibiotics used for lipase-producing selected strains were: ampicillin (0.063–64); chloramphenicol (0.125–256); clindamycin (0.016–32); erythromycin (0.016–32); kanamycin (0.016–32); penicillin G (0.016–32); streptomycin (0.063–64); tetracycline (0.016–32) and vancomycin (0.063–64). The cut-off values for determining MIC of selected strains were based on the the cut-off values as suggested by European Committee on Antimicrobial Susceptibility Testing (Eucast) (2017).

#### *3.2.2.6 PCR detection of staphylococcal enterotoxins (SE) encoding genes*

The detection of classical SE encoding genes (*sea*, *seb*, *sec*, *sed*, and *see*) was performed using the molecular method described by Moura *et al.* (2012). All primers used are summarized in Table 11. The PCR reactions were prepared in a final volume of 25 μL containing 10 pM of each primer, 1 × DreamTaq mastermix, DNase-free water, and approximately template DNA. Amplification of *sea* and *sed* genes was performed under the following thermal cycle condition: 5 min at 94°C; followed by 30 cycles of 45 s at 94°C, 45s at 54°C, and 45 s at 72°C; and final elongation for 5 min at 72°C. The other thermal program to detect the *seb*, *sec*, and *see* genes was as follow: 5 min at 94°C; followed by 35 cycles of 45s at 94°C, 45s at 55°C, and 45s at 72°C; and final elongation for 5 min at 72°C. The amplified products were resolved on 1.5% agarose gel.

#### *3.2.2.7 Biogenic amines production*

The determination of biogenic amines production of selected isolates was performed using TLC and molecular method as previously described in Chapter 2.

## 3.3 Results and Discussions

### 3.3.1 Isolation and screening of lipase-producing bacteria from Kapi

Lipase-producing bacteria were isolated and screened from the first step of Kapi fermentation. Modified M73, MRS, and nutrient agar supplemented with 1% of olive oil were used to screen lipase-producing bacteria. A total of 46 isolates exhibiting orangish clear zone around its colony were obtained. Among them, 10 isolates were selected to determine their lipase activity quantitatively by copper-soap method (Lee and Rhee, 1993). Finally, 4 isolates (LSM3, LSM4, LSM15, and LSM16) exhibiting the highest lipase activity (Table 12) were subjected for further experiments.

**Table 12.** Lipolytic activity of selected lipase-producing bacteria

| Source | Isolate code | Growth medium | Lipase activity (U/mL)* |
|---|---|---|---|
| A | LSM2 | mM73 | 0.29±0.01 |
| B | LSM3 | mM73 | 0.45±0.03 |
| B | LSM4 | mM73 | 0.43±0.02 |
| C | LSM5 | mM73 | 0.37±0.02 |
| C | LSM9 | mM73 | 0.33±0.03 |
| C | LSM10 | mM73 | 0.49±0.04 |
| C | LSM11 | MRS | 0.32±0.02 |
| C | LSM12 | MRS | 0.28±0.01 |
| B | LSM15 | NA | 0.53±0.06 |
| C | LSM16 | NA | 0.66±0.03 |

A, producer A; B, producer B; C, producer C

*Values are given as mean ± SD from triplicate determination

#### *3.3.1.1 Morphology and physiological properties of selected lipase-producing bacteria*

Morphological characterization revealed that all 4 isolates were catalase positive, Gram-positive, and non-spore-forming bacteria. All isolates were able to grow similarly well at 30-40$^{o}$C, while the slow growth was observed at 45$^{o}$C. Isolates LSM3, LSM4, and LSM15 were able to grow well in the presence of NaCl up to 15% while

isolate LSM 16 showed well growth in the presence of 20% of NaCl. In addition, all isolates exhibited poorly growth at concentration higher than 25% of NaCl (Table 13). Salt tolerance is the principal technological properties for the starter development in Kapi production because Kapi is prepared with high salt content about 10-30% (Faithong and Benjakul, 2012).

**Table 13.** Morphology and physiology properties of selected lipase-producing bacteria

| Characteristics | Isolate | | | |
|---|---|---|---|---|
| | LSM3 | LSM4 | LSM15 | LSM16 |
| Gram staining | Positive | Positive | Positive | Positive |
| Catalase test | Positive | Positive | Positive | Positive |
| Spore staining | Non-spore forming | Non-spore forming | Non-spore forming | Non-spore forming |
| Growth in TSB at different temperature (°C)[a] | | | | |
| 30 | ++ | ++ | ++ | ++ |
| 35 | +++ | +++ | +++ | +++ |
| 37 | +++ | +++ | +++ | +++ |
| 40 | ++ | ++ | ++ | ++ |
| 45 | + | + | + | + |
| Growth in TSB at various NaCl concentration (% w/v)[a] | | | | |
| 0 | ++ | ++ | ++ | ++ |
| 5 | +++ | +++ | +++ | +++ |
| 10 | +++ | +++ | +++ | +++ |
| 15 | ++ | ++ | ++ | ++ |
| 20 | + | + | + | ++ |
| 25 | + | + | + | + |
| 30 | - | - | - | - |

[a]The bacterial growth at different temperatures and NaCl concentration is indicated as follows: (+++) means fully (OD600 >1.5); (++) means moderately (OD600 ~ 0.5–1.5); (+) means poorly (OD600 ~0.1–0·5); (-) means no growth (OD600 <0.1).

#### *3.3.1.2 Molecular analysis of the selected strains*

All the selected isolates were also primarily identified by 16S rRNA sequence analysis method. It was found that all isolates showed high similarity to genus *Staphylococcus* with approximately 99% similarity (Table 14). In order to confirm certain species, the specific primers (TstaG422 and Tstag765, Table 11) were used to amplify the identity gene for staphylococci. Based on identification with the specific primers, all selected isolates were identified as staphylococci (Figure 14). In addition, according to 16S rRNA sequence analysis, isolates LSM3 and LSM4 were identified as *Staph. saprophyticus*, while LSM15 and LSM16 were identified as *Staph. xylosus*. All the 16S rRNA sequences were deposited in Genbank, with accesion numbers MG251636, MG251637, MG251638, and MG251639 for isolates LSM3, LSM4, LSM15, and LSM16, respectively. The phylogenetic tree constructed with MEGA v7.0 (Figure 15) also showed that LSM3 and LSM4 were closest in similarity with *Staph. saprophyticus*, while isolate LSM 15 and LSM16 were closest with *Staph. xylosus*.

**Table 14.** Similarity of selected lipase-producing strains based on 16S rRNA sequence analysis

| Strain | Analysis based on 16S rRNA | | |
|---|---|---|---|
| | Closest type strain | Sequence similarity (%) | GenBank accession number of type strain |
| LSM3 | *Staph. saprophyticus* ATCC 15305 | 99 | NR074999.2 |
| LSM4 | *Staph. saprophyticus* ATCC 15305 | 99 | NR074999.2 |
| LSM15 | *Staph. xylosus* ATCC 29971 | 99 | D83374.1 |
| LSM16 | *Staph. xylosus* ATCC 29971 | 99 | D83374.1 |

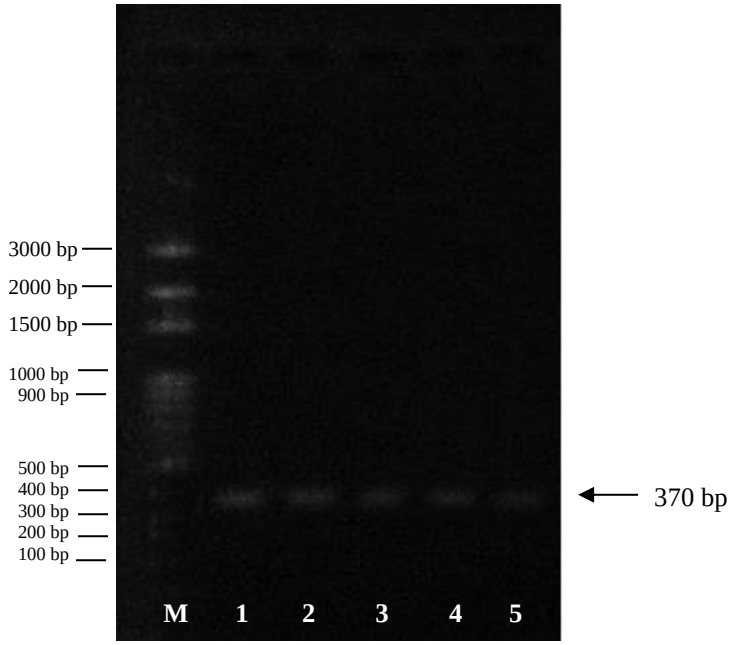



**Figure 14.** The PCR products with staphylococci housekeeping genes for selected strains. Lane M: 100bp DNA ladder; lane 1: *Staph. aureus* DMST8840; lane 2: LSM3; lane 3: LSM4; lane 4: LSM15; lane 5: LSM16.

*Staph. saprophyticus* can be found in raw beef and pork, dairy product, cheese, dry fermented sausages, also in food utensils used in food processing (Coton *et al.*, 2010; Rall *et al*., 2010; Soares *et al*., 2011). This Gram-positive coccus is the second main responsible for uncomplicated lower urinary tract infections (UTIs) after *E. coli* (Raz *et al*., 2005). Meanwhile, *Staph. xylosus* which was also found in many fermented meat products and their derivatives, was rarely found to be pathogenic as *Staph. saprophyticus*, so that *Staph. xylosus* can be widely applied as starter culture (Jeong *et al*., 2016).

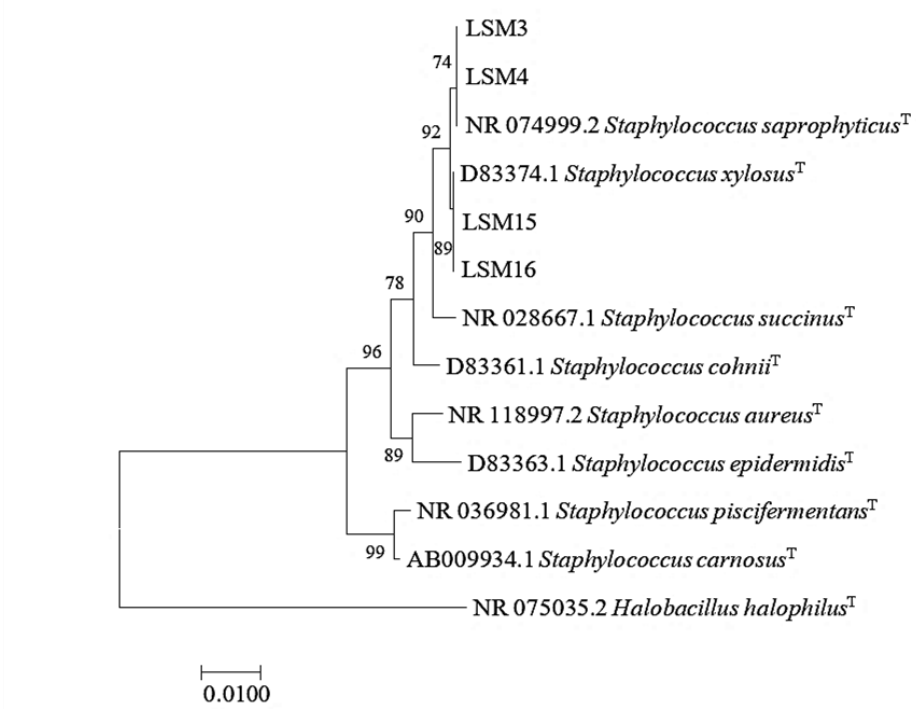



**Figure 15.** Phylogenetic tree of the type strain of *Staphylococcus* species and selected lipase-producing bacteria isolated from Kapi. The tree is constructed based on the results of 16S rRNA sequencing. The scale bar corresponds to ten nucleotide substitutions per 1000 nucleotides (evolutionary distances). Numerals indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test which are shown next to the branches. Evolutionary analyses were conducted in MEGA7.

### 3.3.2 Technological properties and safety assessments of the selected strains

#### *3.3.2.1 Proteolytic activity*

Certain technological properties should be possessed by a good starter culture in order to adapt the condition during food fermentation. Together with lipase activity that exhibit by each strain in this study, the protease activity of each strain was also investigated in skim milk and casein agar plates. Nevertheless, none of the isolates showed proteolytic activity. The same report was obtained by Jeong *et al.* (2016) who found that most *Staph. saprophyticus* and *Staph. xylosus* strains isolated from fermented

soybean possessed lipolytic activity did not exhibit protease activity however, only two *Staph. saprophyticus* strains and a *Staph. xylosus* strain exhibited strain-specific protease activity. In other studies, *Staph. xylosus* strains isolated from fermented sausages showed esterase–lipase activities (Garcĭa-Varona *et al.*, 2000; Papamanoli *et al.*, 2002). However, little information about the protease production has been reported for *Staphylococcus* isolated from seafood fermentation. In fact, bacterial proteases would also make available the formation of flavor in the fermented food (Martín *et al.*, 2007) besides the primary lipolysis which contributed to flavor development in fermented food (Jeong *et al.*, 2016).

#### *3.3.2.2 Antimicrobial activity*

Since the application of starters having antimicrobial potency against pathogenic microorganisms has acknowledged greatly in fermented food product, the antimicrobial activity against pathogenic bacteria is one important functional characteristics of starter cultures needs to be investigated (Drosinos *et al.*, 2005). Thus, the antimicrobial activity of the selected strains was investigated against the most frequently found pathogens, e.g. *B. cereus*, *E. coli*, *L. monocytogenes*, *Staph. aureus*, *Sal.* Typhimurium, and *V. parahaemolyticus*. It was found that *Staph. saprophyticus* LSM4, *Staph*. *xylosus* LSM15, and *Staph*. *xylosus* LSM16 exhibited antimicrobial activity toward only Gram-positive indicator bacteria *B. cereus* DMST5540, *Staph. aureus* DMST8840, and *L. monocytogenes* DMST17303 (Table 15). None of the Gram-negative bacteria (*E. coli*, *V. parahemolyticus*, and *Sal.* Typhimurium) were inhibited by isolated strains. This is because the cytoplasmic membrane of Gram-negative bacteria is protected by an outer membrane composed of a phospholipid bilayer surrounded by a tight shield of lipo-polysaccharide layer that hardly to be attacked by many substances or compounds, such as bacteriocins, antibiotics, hydrophobic compounds, detergents, and dyes (Raetz and Whitfield, 2002; Vaara, 1992). The similar results were reported by Villani *et al.* (1997), Papamanoli *et al*. (2002), and Martín *et al.* (2007), which showed that strains of *Staph. xylosus* displayed antimicrobial activity against *Staph. aureus* and *L. monocytogenes*. However, none of those reports explained about the origin of antimicrobial exhibited by the strains of *Staph. xylosus*.

The antibacterial origin of the *Staph. saprophyticus* LSM14, *Staph. xylosus* LSM15, and *Staph. xylosus* LSM16 were then further studied (Table 16). The CFS which was directly used exhibited the inhibitory effect to *Staph. aureus* DMST8840 as indicator strain. This inhibition effect might probably due to the production of antibacterial compounds, such as organic acids or bacteriocin. After ruling out the effects of organic acids (CFS-N), all the strains remain unchanged activity in inhibiting the indicator strain. This could indicate that all the strains produced the bacteriocin-like inhibitory substance (BLIS). Moreover, when the CFS-N treated with proteolytic enzymes (CFS-NP), the complete inactivation was observed. This result provides the preliminary assumption that the inhibitory activity of the selected strains originated not from the organic acids action but from proteinaceous substances such as BLIS. Some bacteriocins produced by *Staph. xylosus* have been categorized belong to class IId lactococcin-like bacteriocins (Carson *et al*., 2017). However, so far none of the *Staph. xylosus* isolates have been characterized for its bacteriocin encoding genes. Thus, to conduct the PCR methods, the primers used were designed based on the nukacin ISK-1 structural gene (*nukA*) and the post-translational modification encoding gene for nukacin (*nukM*) of *Staph. warneri* and *Staph. pasteuri* (Sashihara *et al*., 2000; Hong *et al*. 2014). This nukacin ISK-1 belongs to the same class IId lactococcin-like bacteriocins of *Staph. xylosus* (Carson *et al*., 2017). Hong *et al*. (2014) reported that only nukacin ISK-1 structural gene *nukA* could be amplified for *Staph. pasteuri*, but *nukM* could not be amplified. In the present study, none of the genes encoding nukacin bacteriocin could be amplified using those selected primers. This is probably due to the fact that those primers were not specific for the bacteriocins of *Staph. xylosus*. Further study about the bacteriocins and genes encoding them should be performed to define the bacteriocins of *Staph. xylosus*.

**Table 15.** Antimicrobial activity of selected strains toward several pathogenic bacteria by agar spot assay

| Indicator pathogenic bacteria | Isolate | | | |
|---|---|---|---|---|
| | LSM3 | LSM4 | LSM15 | LSM16 |
| *Staph. aureus* DMST8840 | - | ++ | ++ | ++ |
| *E. coli* DMST4212 | - | - | - | - |
| *B. cereus* DMST5540 | - | + | + | + |
| *V. parahemolyticus* DMST5665 | - | - | - | - |
| *Sal.* Typhimurium DMST 5784 | - | - | - | - |
| *L. monocytogenes* DMST17303 | - | ++ | ++ | ++ |

Notes: (+++) showed clear zone >20mm; (++) showed clear zone 11-20mm, (+) showed clear zone <10mm; (-) no activity.

**Table 16.** The inhibitory spectrum of antibacterial compounds produced by selected strains to *Staph. aureus* DMST8840 as indicator strain

| Isolate | Inhibitory activity | | | | | |
|---|---|---|---|---|---|---|
| | CFS | CFS-N | CFS-NP | | | |
| | | | α-chy | Pep | Pro K | Try |
| *Staph. saprophyticus* LSM4 | ++ | ++ | - | - | - | - |
| *Staph xylosus* LSM15 | ++ | ++ | - | - | - | - |
| *Staph xylosus* LSM16 | ++ | ++ | - | - | - | - |

Notes: (+++) showed clear zone >20mm; (++) showed clear zone 11-20mm, (+) showed clear zone <10mm; (-) no activity.

α-chy: α-chymotrypsin; Pep: pepsin; Pro K: proteinase K; Try: trypsin

#### *3.3.2.3 Hemolytic activity*

In the present study, hemolytic activity of selected strains was also investigated. Hemolytic activity has been linked to virulence in many pathogenic microorganisms because some bacteria have developed hemolytic activity to obtain nutrients from red blood cells (Vela *et al.*, 2012). This will cause a human's health at

risk by consuming the food contaminated with those bacteria. Therefore, strains exhibited hemolytic activity may not be applicable as starter candidates. Our results revealed that isolates LSM3, LSM15, and LSM16 displayed non-hemolytic activity (γ-hemolysis), meanwhile isolate LSM4 displayed α-hemolytic activity (Table 17). Jeong *et al.* (2016) reported that 18.4% of 49 strains *Staph. saprophyticus* isolated from fermented soybean displayed α-hemolytic activity, while 46.9% displayed γ-hemolytic activity. In addition, they also reported that 66.7% of 18 strains *Staph. xylosus* displayed γ-hemolytic activity and 11.1% displayed α-hemolytic activity. These results suggested that hemolytic activity is a strain-dependent characteristic (Marty *et al.*, 2012). Lipase-producing strains displayed γ-hemolytic activity can be considered as the strain which did not induce hemolysis (Oh and Jung, 2015) and may be safe to be used as starter culture.

**Table 17.** Hemolytic activity and biofilm formation ability of selected lipase-producing bacteria

| Isolate code | Hemolytic activity[a] | Biofilm formation ability[b] | PCR result for biofilm formation ability[c] |
|---|---|---|---|
| *Staph. saprophyticus* LSM3 | - (γ) | W | - |
| *Staph. saprophyticus* LSM4 | + (α) | W | - |
| *Staph. xylosus* LSM15 | - (γ) | W | - |
| *Staph. xylosus* LSM16 | - (γ) | W | - |
| *Staph. aureus* DMST4212 | + (β) | M | + |

[a] + (α) means the isolate showed α-hemolysis activity; + (β) means the isolate showed β-hemolysis activity; - (γ) means the isolate showed γ-hemolysis activity;

[b] M means moderate activity, W means weak ability

[c] (+) showed band correspond with the DNA fragment of *ica*A targeting gene; (-) no band showed correspond with the DNA fragment of *ica*A targeting gene

#### *3.3.2.4 Biofilm formation ability*

Biofilm formation ability is a phenomenon which happens in microorganisms and surfaces exist in close proximity (Hall-Stoodley *et al.*, 2012). In the food industry, the microbial attachment to various surfaces may contribute to potential hazard. Biofilm production has been reported also as one of the pathogenesis indicators by the staphylococci (Jain and Agarwal, 2009). This could be later involved in various microbial infections in the human body through the food consumption (Shi and Zhu, 2009).

In this study, all staphylococci strains displayed weak biofilm formation which was determined spectrophotometrically based on the criteria proposed by Stepanovic *et al.* (2000). Confirmation test were also performed by PCR amplification of *icaA* gene which is responsible in biofilm formation. It was found that all strains were *icaA* negative (Table 17). This result was in accordance with Landeta *et al.* (2013) who found that *Staph. xylosus* strains which showed weak biofilm formation detected spectrophotometrically also were *ica*A negative. The presence of the *ica*A was positive only in some strains that previously were positive as determined by spectrophotometric method. This correlation can be found in the case of *Staph. aureus* which showed moderate biofilm formation ability determined by spectrophotometric method and also showed positive band of *ica*A. This result indicates that biofilm formation ability could be considered as strain dependent characteristic (Jeong *et al.*, 2014). In addition, the existence of genes probably involve in the bifilm formation is one indicator for confirming biofilm formation ability of tested strains (Landeta *et al*., 2013; Asai *et al*., 2015).

#### *3.3.2.5 Antibiotic susceptibility*

In the selection of promising starter culture, antibiotic resistance is one important characteristic should be considered first. This is because starter culture can be the potential contributing vector of antibiotic to microbiota through the fermentation process. These determinants can spread to humans through the food consumption (CDC, 2013). In this study, antibiotic susceptibility of all selected lipase-producing strains was tested against nine generally human used antibiotics including ampicillin,

chloramphenicol, clindamycin, erythromycin, kanamycin, penicillin G, streptomycin, tetracycline, and vancomycin. It was found that strains LSM15 and LSM16 showed sensitivity to all antibiotics tested. However, isolate LSM3 showed resistance to clindamycin and LSM4 resisted to both clindamycin and tetracycline (Table 18). *Staph. saprophyticus*, which is the second pathogenic bacteria after *E. coli* commonly caused urinary tract infection so that the incidence to find resistance in most *Staph. saprophyticus* against numerous antibiotics, including those of clinical importance are common (Price and Flournoy, 1982; Kuroda *et al.*, 2005). Podkowic *et al*. (2012) reported that two strains of *Staph. saprophyticus* isolated from ready-to-eat meat harbored the *tet* (tetracycline resistance gene). In addition, Lee *et al.* (2015) also reported that *Staph. saprophyticus* strains isolated from Korean fermented soybean paste showed resistance to tetracycline and lincosamide antibiotics (such as clindamycin). Antibiotic resistance in staphylococci may cause health problem in humans because they may contribute in nosocomial and bloodstream infections (Osman *et al.*, 2016).

**Table 18.** Antibiotic susceptibility of selected lipase-producing bacteria

| Antibiotics[a] | Cut-off value ($\mu g\ mL^{-1}$)[b] | $MIC_{90}$ of isolate | | | |
|---|---|---|---|---|---|
| | | LSM3 | LSM4 | LSM15 | LSM16 |
| Amp | n.r.[c] | 0.5 | 4 | 0.13 | 0.25 |
| Cam | 16 | 8 | 8 | 8 | 1 |
| Clin | 0.26 | 0.5[d] | 0.5[d] | 0.13 | 0.13 |
| Ery | 2 | 1 | 0.26 | 0.06 | 1 |
| Kan | 2 | 0.26 | 0.06 | 0.13 | 0.26 |
| Pen G | 2 | 1 | 1 | 1 | 0.13 |
| Str | n.r.[c] | 4 | 8 | 2 | 0.26 |
| Tet | 2 | 0.5 | 4[d] | 0.5 | 0.5 |
| Van | 4 | 2 | 4 | 2 | 2 |

[a] Amp, ampicillin; Cam, chloramphenicol; Cli, clindamycin; Ery, erythromycin; Kan, kanamycin; Pen G, penicillin G; Str, streptomycin; Tet, tetracyclin; and Van, vancomycin

[b] The cut-off values as suggested by Eucast (2017)

[c] n.r.: not required

[d] showed resistance to corresponding antibiotics

#### *3.3.2.6 Staphylococcus enterotoxin (SE) genes*

One of the causes of food poisoning is the ability of some microorganisms to produce toxin in the food processing. The genus *Staphylococcus* is overly concerned about its pathogenicity because they can produce staphylococcal enterotoxin (SE) lead to food poisoning. SE is a type of emetic toxins and is generally classified as one of the members of the pyrogenic toxin superantigen family (Balaban and Rasooly, 2000). Several SE genes have been found in staphylococci, but the most frequently reported (as classical SE genes) are *sea, seb, sec, sed,* and *see*. Because of SE's pathogenicity, the staphylococci intended to be applied as starter culture should not harbor the SE genes. In this study, amplification of SE genes was not observed from any of selected strains suggesting that they would not produce SEs (Table 11). The pathogenic *Staph. aureus* was the most staphylococci harboring the SE genes compared

with others (Udo *et al.*, 1999; Blaiotta *et al.*, 2004). Meanwhile, the most prevalent coagulase-negative staphylococci (CNS) harbored the SE genes are *Staph. epidermidis* and *Staph. cohnii* while *Staph. xylosus* is rarely reported (Guimaraes *et al.*, 2013; Piechota *et al.*, 2014). Our study revealed that all the isolated strains did not harbor any of the classical SE genes which means they are safe to be applied as starter cultures in the Kapi production.

#### *3.3.2.7 Biogenic amine production*

Another key factor related to the use of staphylococci affecting food safety is their ability to produce BAs. BAs can be found widely in fermented food products especially those with high protein content (Shalaby, 1996; Sila Santos, 1996). The activity of decarboxylation of amino acids by various microbes can increase the levels of BAs in food products. This can lead to contaminated foodstuffs and endangered the safety of humans (Naila *et al.*, 2010). However, information on the production of BAs is limited to staphylococci isolated from fermented meat or sausages. Very few reports are currently available regarding the ability of BAs production in staphylococci isolated from seafood fermentation. In this study, *Staph. xylosus* LSM16 was selected for determining its ability in BAs production. It was found that this strain did not produce the four BAs (histamine, putrescine, cadaverine, and tyramine) by TLC method as depicted in Figure 16. In addition, based on PCR method, it was found that *Staph. xylosus* LSM16 did not harbor the *hdc*, *tdc, odc,* and *ldc* genes (Table 10). Seitter *et al.* (2011) reported that the formation of BAs has not been found in *Staph. xylosus* isolated from various fermented foods. The most common BAs found in fermented foods and microorganisms related to them are histamine and tyramine. These BAs have toxicological effects causing vasodilation and affecting the central nervous system (Blough *et al.* 2014). In addition, putrescine and cadaverine have toxic effects because of their high relevancy with histamine and tyramine (Perin *et al*., 2014). Talon and Leroy (2011) considered BAs as major concerns because they can lead to food poisoning. Several coagulase-negative staphylococci (CNS) such as *Staph. carnosus, Staph. epidermidis, Staph. piscifermentans, Staph. saprophyticus, Staph. warneri,* and *Staph*. *xylosus* can decarboxylate amino acids (de las Rivas *et al.*, 2008). However, the

specifity, presence, as well as the activity of decarboxylases are strain-specific (Belicová *et al*., 2013). In the present study, it has been found that *Staph. xylosus* LSM16 can be regarded as non-BA producer as confirmed by TLC and molecular methods. Therefore, this strain is safe to be applied as a starter culture in the Kapi production.

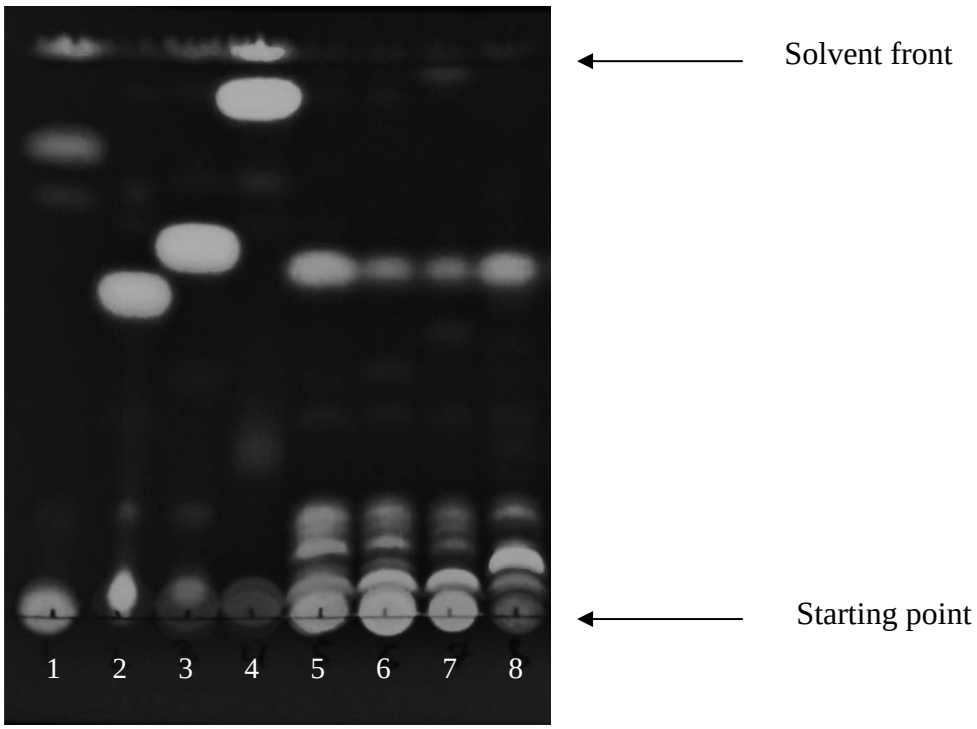



**Figure 16.** Dansylated biogenic amines standards as separated by TLC: histamine (1), putrescine (2), cadaverine (3), and tyramine (4). Culture-free supernatants of *Staph. xylosus* LSM16 grown in TSB medium containing substrate: histidine (5), ornithine (6), lysine (7), tyrosine (8).

### 3.4 Conclusions

*Staph. xylosus* LSM16 showed remarkably technological properties to be applied as a starter culture for the Kapi production. It exhibits high lipase activity, considerable salt tolerance, has BLIS activity toward pathogenic bacteria, γ-hemolysis, weak biofilm formation ability, and susceptible to all antibiotics tested. In addition, it was also did not harbor the classical SE genes and genes encoding several important

decarboxylase enzymes. All these features are principal factors that should be possessed by a good and functional starter culture. Therefore, the *Staph. xylosus* LSM16 could be applied as the promising starter culture in the Kapi production. Application of this strain might be contributing to improvement of Kapi quality.

# CHAPTER 4

## OPTIMIZATION OF PROTEASE PRODUCTION BY *Virgibacillus halodenitrificans* MSK-10P


### Abstract

The aim of this study was to determine the optimum conditions for the production of protease by *Virgibacillus halodenitrificans* MSK-10P isolated from Kapi. This strain was selected not only because it exhibited high protease activity among five isolated protease-producing bacteria, but it also had several important technological properties and safety attributes. A one time-factor-at a-time (OFAT) method was employed to find out the parameter affecting its protease production. Temperature and pH optimum for the protease production by *V. halodenitrificans* MSK-10P were 37$^{o}$C and 8.0, respectively. In addition, casein (1%, w/v) was suitable for high protease production. Moreover, the maximum protease production was obtained by 2.5% (v/v) of inoculum size, agitation at 150 rpm, and the addition of 10% (w/v) NaCl in the medium. The obtained crude extract of the protease was stable at 0-25% of NaCl. Cultivation under optimum condition revealed that the highest protease production was obtained at 36 h incubation time. The activity was increased 3.83-fold from 9.47 U/mL to 36.32 U/mL after optimization of culture condition. Therefore, this protease from strain MSK-10P has potential for application in food processing that needs high salt condition.


Keywords: halotolerant, Kapi, optimization, protease, *Virgibacillus halodenitrificans* MSK-10P.

## 4.1 Introduction

Proteases hydrolyze peptide bonds in proteins into small chain peptides and free amino acids which all living cells can captivate and utilize these smaller molecules easier. Proteases are widely applied in the industrial processing required protein degradation since they have broad specificity of the substrate, especially in the food and beverage, textile, pharmacy, and chemicals industries. Proteases also account for about 60% of global market for enzyme application (Sarrouh *et al*., 2012). The enzyme-based process technology potentials to use raw materials efficiently, produce minimal or no waste, and avoid the use of toxic chemicals (Singh and Bajaj, 2016).

Halophilic enzymes have inimitable enzymatic purposes compared with non-halophilic. Halophilic enzymes can remain their higher activity and longer stability even in the environment with high salt concentrations (1–4 M). Meanwhile, halotolerant protease has unique catalytic purposes. It can function at wide range of salt environment, from no salt until high salt condition (Amoozegar *et al*., 2003; Suganthi *et al*., 2013).

Most of the fishery fermentation carried out in high salt condition. Kapi was produced in the salt concentration around 10-30% (w/w) NaCl (Faithong *et al*., 2010). Halophilic and halotolerant microorganisms which contributed to the shrimp paste fermentation process, such as *Bacillus* sp., *Lentibacillus* sp., *Pediococcus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Tetragenococcus* sp., *Virgibacillus* sp., were reported as the source of protease involved in the fermentation process (Surono and Hosono, 1994; Kobayashi *et al*., 2003; Pakdeeto *et al*., 2007; Chuon *et al*., 2013; Lestari *et al*., 2017).

*Virgibacillus* sp. has been classified as halotolerant group of bacteria mostly found in saline habitats including food matrices (Heyrman *et al*., 2003). Several species of this genus are able to produce extracellular proteases. *V. halodenitrificans* SK1-3-7 which was isolated from Thai fish sauce has been found to produce fibrinolytic enzymes (Montriwong *et al*., 2012). Serine alkaline proteases also were characterized from *V. pantothenticus* (MTCC 6729) and *V. dokdonensis* VITP14 (Gupta *et al*., 2008; Rajeswari *et al*., 2012). In addition, the protease from *V. halodenitrificans* RSK CAS1

was characterized as thermostable, halo-alkaline, as well as high stability to solvents and detergents (Sathishkumar *et al*., 2014).

In this study, *V. halodenitrificans* MSK-10P isolated from Kapi which previously selected was investigated for its protease production based on its technological properties and safety attributes, such as high proteolytic activity, tolerate to the high salt environment, γ-hemolysis, susceptible to several antibiotics tested, and neither a biofilm nor BAs producer. Several physical and chemical conditions required for the production of protease was optimized using one-factor-at a-time (OFAT) method to determine the optimum condition for achieving high protease production.

## 4.2 Materials and Methods

### 4.2.1 Bacterial strain and inoculum preparation

A loopful of *V. halodenitrificans* MSK-10P strain after growth on NA plate at 37 °C for 2 days was inoculated in a flask containing 25 mL of nutrient broth (NB, HiMedia, Mumbai, India) medium supplemented with 10% (w/v) NaCl. The flask was incubated at 37 °C under agitation at 150 rpm for 24 to 36 h. The cell culture was used as an inoculum when its optical density at 600nm (OD600) was around 1.0. Then, 1% (v/v) of inoculum was transferred into enzyme production medium. Production medium (PM) was a modified medium by Aruna *et al*. (2014) consisted of (%w/v): peptone 1.0; beef extract 1.0; NaCl 10.0; and skim milk 1.0, with pH set at 7.0. Culture broth of *V. halodenitrificans* MSK-10P was harvested by centrifugation (9800 × *g*) at 4°C for 10 min and used as crude protease for the determination of protease activity.

### 4.2.2 Optimization of protease production by *V. halodenitrificans* MSK-10P

The one-factor-at a-time (OFAT) experiment was used to determine the optimum condition for protease production. Initial conditions were as follows: pH medium was 7.0, time of incubation was 24 h, and agitation speed was 150 rpm.

#### *4.2.2.1 Effect of incubation temperature on protease production*

The optimum temperature for protease production was investigated by culturing the strain in PM at various temperatures (25–50 °C), under shaking at 150 rpm. The protease production was determined in terms of protease activity by using modified Anson method as previously described in Chapter 2.

#### *4.2.2.2 Effect of initial pH on protease production*

The optimum of initial pH on protease production was investigated by culturing *V. halodenitrificans* MSK-10P in PM at various pH values: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. The pH of the medium was adjusted using 0.1N HCl or 0.1N NaOH. The cultivation was performed at selected optimum temperature obtained from section 4.2.2.1 for 24 h with constant agitation at 150 rpm. Then, the protease production was measured.

#### *4.2.2.3 Effect of additional nitrogen source on protease production*

The effect of different nitrogen sources on the protease production was investigated by incorporating the available nitrogen source in PM (peptone and beef extract) with 1% (w/v) of various additional nitrogen source including casein, skim milk, bovine serum albumin (BSA), shrimp shell powder (SSP), tuna condensate (TC), and urea. The SSP was prepared by cleaning the shrimp shell with water, drying for 12 h at 55$^{o}$C, and grinding the shrimp shell through a 22-mesh sieve to obtain the SSP. The TC was prepared by freeze-drying the tuna condensate from the local tuna canning factory. Furthermore, the effect of different concentrations of the selected additional nitrogen source (0.5, 1.0, 1.5, 2.0, and 2.5%) was also investigated. The cultivation was performed with the optimum temperature and pH as selected from section 4.2.2.2 and 4.2.2.3 under shaking at 150 rpm for 24 h. Afterwards, the protease production was measured.

#### *4.2.2.4 Effect of optimum NaCl concentration on protease production and stability*

The effect of various NaCl concentrations on the protease production was also investigated. Various NaCl concentrations (0.0, 3.0, 5.0, 7.0, 10.0, 15.0, 20.0,

25.0, and 30.0 %, w/v) were added into PM containing selected conditions from previous experiment. The protease activity was determined after incubation for 24 h with shaking speed at 150 rpm.

In addition, the stability of protease under various NaCl concentrations was also investigated. Crude protease was pre-incubated in phosphate buffer (50 mM, pH 8.0) containing various NaCl concentrations (% w/v) of 0.0, 3.0, 5.0, 7.0, 10.0, 15.0, 20.0, 25.0, and 30.0, for 2 h at 37$^{o}$C. Afterwards, the activity was mesured by using modified Anson method as previously described and the relative activity of crude enzyme was determined using the maximum activity of the respective NaCl concentration as 100% (Sinha and Khare, 2012).

#### *4.2.2.5 Effect of inoculum concentration on protease production*

The effect of the inoculum concentrations on protease production was also investigated. Various concentrations of bacterial culture ((% v/v): 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, and 10.0) were added to the medium with the optimum NaCl concentration. The protease activity was determined after incubation for 24 h under previously selected conditions.

#### *4.2.2.6 Effect of agitation speed on protease production*

The optimum agitation speed (100, 150, 200, and 250 rpm) required for protease production was investigated. The protease activity was determined after incubation for 24 h under previously selected conditions.

### 4.2.3 Time course of protease production

For the time course study of the protease production, the combination of selected conditions and medium from sub-section 4.2.2.1-4.2.2.6 was used. The bacterial culture with optimal inoculum concentration was transferred into 50 mL of PM containing suitable additional nitrogen source and concentration of NaCl with optimum initial pH. The culture was incubated at selected temperature and agitation for given period (72 h). The samples were taken continuously at 6 h of the time interval until 72 h for measuring the cell growth and protease production. The cell growth was

measured by using spread-plate method on NA plate supplemented with 10% (w/v) NaCl and 1% (w/v) casein.

### 4.2.4 Protease activity assay

Protease activity was determined according to modified Anson method developed by Cupp-Enyard (2008) as previously described in Chapter 2, sub section 2.1.2.

### 4.2.5 Statistical analysis

All the experiments were performed in triplicate and level of significance was ± 5%. Statistical analysis was performed using SPSS v 16.0. Analysis of Variance (ANOVA) and mean comparison by Duncan's Multiple Range Test (DMRT) were performed.

## 4.3 Results and Discussions

### 4.3.1 The optimization of protease production

#### *4.3.1.1 Effect of temperature on protease production*

The effect of temperature on protease production by *V. halodenitrificans* MSK-10P can be figured out in Figure 17. In this experiment, the highest protease activity of 12.03 U/mL was obtained at 37$^{o}$C, followed by at 40$^{o}$C which gave slightly different activity of 11.36 U/mL ($p$<0.05). These incubation temperatures gave activities higher than that the initial activity (9.47 U/mL) performed at 30$^{o}$C. However, the decrease in activity was found at high temperature of 45 and 50$^{o}$C which gave lower protease activity of 10.23 and 7.95 U/mL, respectively. Other reports found the optimum temperature for protease production by *V. pantothenticus* and *V. halodenitrificans* RSK CAS1 was at 40$^{o}$C (Gupta *et al*., 2008; Satishkumar *et al*., 2014).

In this experiment, lower enzyme activity was observed at below optimum temperature (37$^{o}$C) due to low activation energy which are available, while at the temperature 40°C and above, the protease activity decreased. At low temperature, the enzymatic reaction is slow, the increase in temperature will accelerate the reaction, until the optimum temperature is reached, and the enzymatic reaction reaches its

maximum. The increase in temperature over the optimum temperature will cause the enzyme to denature and decrease the speed of enzymatic reactions as well as reduce its activity (Whitaker, 1994).

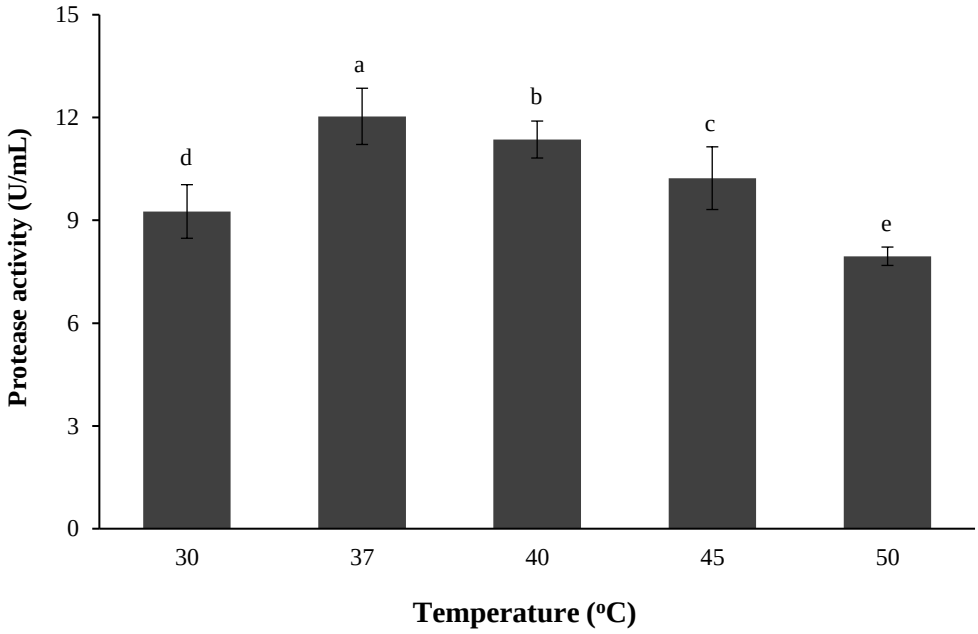



**Figure 17**. Effect of the optimum incubation temperature on protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in PM (pH 7.0) under shaking at 150 rpm for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

#### *4.3.1.2 Effect of initial pH on protease production*

Effect of initial pH on protease production by *V. halodenitrificans* MSK-10P was investigated. Various pH was tested at the range of 4.0 – 11.0 (Figure 18). This strain exhibited protease production increasingly up to a pH of 8.0 and a decreasingly thereafter. The highest protease production was obtained at pH 8.0 (17.18 U/mL), which was almost 2-fold higher than that the initial activity (9.47 U/mL) at pH of 7.0. Similar result was reported by Rajeswari *et al*. (2012) who showed that pH 8.0 was the optimum pH for protease production by *V. dokdonensis* VITP14. Different results were reported by other studies which obtained the pH optimum of protease production by *V. pantothenticus* MTCC 6729 and *V. halodenitrificans* RSK CAS1 was 9.0 (Gupta *et al*., 2008; Satishkumar *et al*., 2015).

It is well understood that the pH of culture media influences the availability of metabolic ions and the permeability of certain bacterial cell membranes, which give effect on the stability or conformation of the plasma membrane. This consequently affects the membrane-bound ribosomes involved in the synthesis or the production of enzymes (Razak *et al*., 1997; Ellaiah *et al*., 2002). The enzyme production could be reduced beyond the optimum pH because of enzyme inactivation or its instability. Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum (Sabu *et al*., 2005).

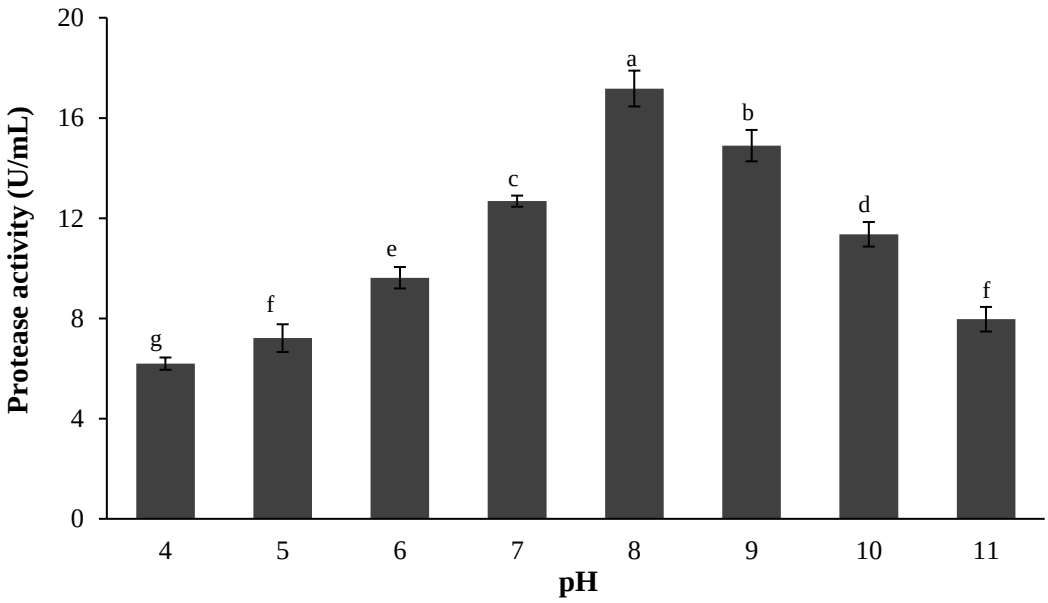



**Figure 18**. Effect of the initial pH on protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in PM under shaking (150 rpm) at 37ºC for 24 h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

#### *4.3.1.3 Effect of additional nitrogen source on protease production*

The effect of additional nitrogen sources as substrate for incorporating available nitrogen source within the production medium on protease production by *V. halodenitrificans* MSK-10P was investigated. Among the tested nitrogen sources (Figure 19), it was found that the presence of casein exhibited the highest protease

production (23.85 U/mL). This result showed 2.5-fold higher than the initial protease activity (9.47 U/mL). In the presence of other nitrogen sources observed in this research, such as skim milk, shrimp shell powder, tuna condensate, BSA, and urea, the protease production was 17.18, 14.26, 15.34, 12.40, and 11.0 U/mL, respectively. Similar report by Park *et al* (2013) showed that the highest protease production was obtained by applying combination of yeast extract and casein. It was also reported by others that using casein as a nitrogen source or as a substrate in protease production gave highest activity (Yang *et al*., 2000; Vijayaraghavan *et al*., 2014). The amino acids and peptides in casein may be served as specific inducers for increasing protease production by *V. halodenitrificans* MSK-10P compared with other nitrogen sources tested in this study (Andrade *et al*., 2002). Therefore, casein was selected to be used as the additional nitrogen source for the next optimization process. Various concentration of casein was optimized for protease production. The optimum concentration of casein was 1% giving the highest activity of 23.89 U/mL (Figure 20). Increasing the casein concentration higher than 1% gave the adverse effect on protease production. This was because the more the substrate level could lead to the suppressive role of substrate and excessive amount of amino acids and nitrogen source which may cause the decreasing of protease production (Chu, 2007).

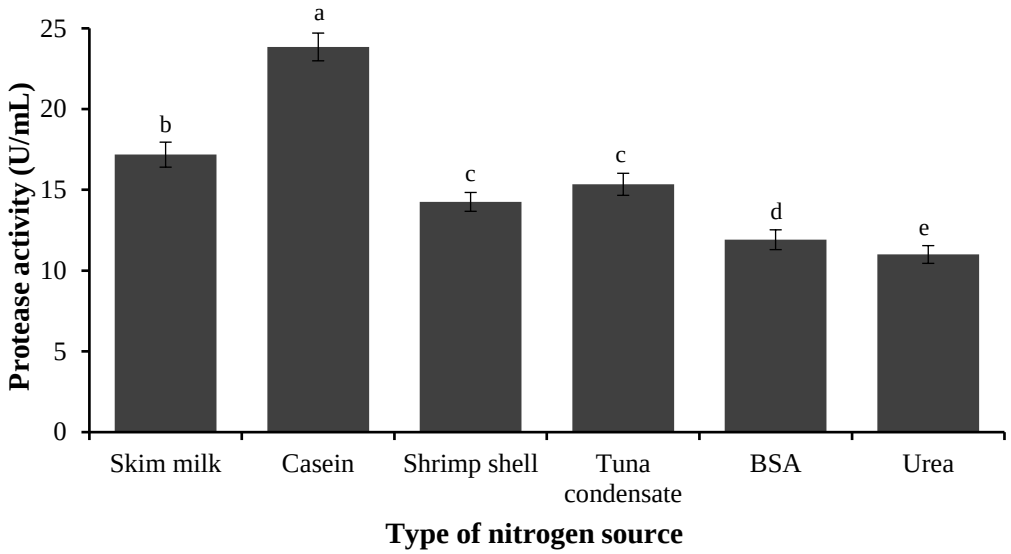



**Figure 19**. Effect of nitrogen source on protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in PM (without skim milk, pH 8.0) under shaking at 150 rpm for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

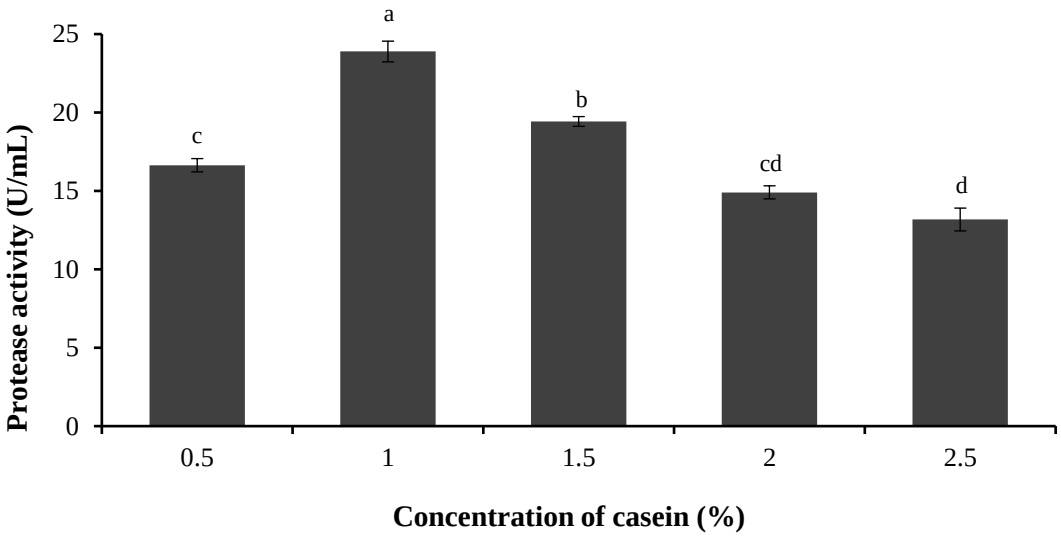



**Figure 20**. Effect of the concentration of casein (%) on protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in PM (pH 8.0) under shaking at 150 rpm for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

#### *4.3.1.4 Effect of NaCl concentration on protease production*

*V. halodenitrificans* MSK-10P was previously identified as a halotolerant. Thus, the effect of various NaCl concentration on protease production by *V. halodenitrificans* MSK-10P was also investigated. It has been found that protease production increased with increasing salinity (0–7 % NaCl, w/v) and reached its maximum at 10 % NaCl (Figure 21). Increasing NaCl higher than 10% caused decreasing of protease production. At optimum NaCl concentration (10%), the production (24.03 U/mL) showed 2.5-fold higher than initial production (9.47 U/mL). Since at the initial condition, several other parameters have not been optimized yet, even the initial NaCl concentration was also 10% (w/v). Similar result has been found by Lam *et al*. (2018) who reported that 10% NaCl was the optimum NaCl concentration for protease production by *Virgibacillus* sp. CD6. The higher NaCl concentration away from optimum point the lower decreasing production obtain. It can be explained that increasing the concentration of salt means increasing the number of ions in solution which lead to imbalancing the electrostatic ions out of optimum concentration needed (Choudhury, 2009). This imbalance may disrupt the metabolism process in bacteria for synthesizing proteins (Wiley *et al*., 2008).

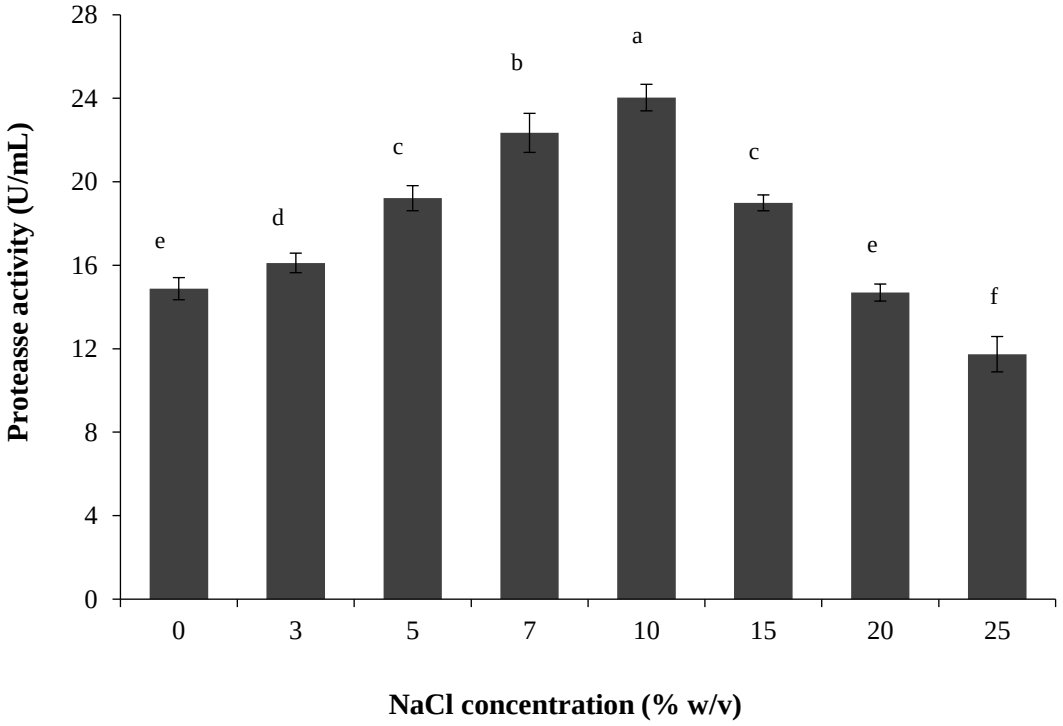



**Figure 21**. Effect of NaCl concentration on protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in modified PM (pH 8.0) under shaking at 150 rpm for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

The stability of crude protease from *V. halodenitrificans* MSK-10P on various NaCl concentrations was also investigated at 0-30% NaCl (w/v). It can be seen from Figure 22 that the protease activity of crude enzyme was stable at wide range of applied NaCl concentration (0-25%). The optimum activity was obtained at 10% NaCl. At 0-20% NaCl concentration, the protease activity still retained its activity above 80%, while at 25% NaCl the activity still retained 75%. At 30% NaCl the activity lost about 50% compared with the highest activity obtained at 10% NaCl concentration. Therefore, it can be concluded that crude protease from *V. halodenitrificans* MSK-10P could work effectively and stable at broad range of NaCl concentration (0-25%). This halostable activity allowed protease from *V. halodenitrificans* MSK-10P to be

potentially useful in food fermentation which required non-salty and salty environment. Other reports found that protease from *Virgibacillus* sp. EMB13 was stable at 0-15% NaCl (Sinha and Kare, 2013), while the protease from *Virgibacillus* sp. CD6 was stable at 0-12.5% NaCl (Lam *et al*., 2018). Protease from other *Virgibacillus* sp. SK33 also exhibited similar stability at various NaCl concentration (Sinsuwan *et al*., 2010). It was clearly shown that halophiles accumulate salt or organic molecules to maintain their cellular osmotic balance. Their enzymes are accustomed to being optimally active at high salt environment condition. Therefore, salt is a crucial factor for protease production by halotolerant microorganisms for maintaining optimum enzymatic activity as well as preventing the protein denaturation (Ventosa *et al*., 1998; Oren, 2008).

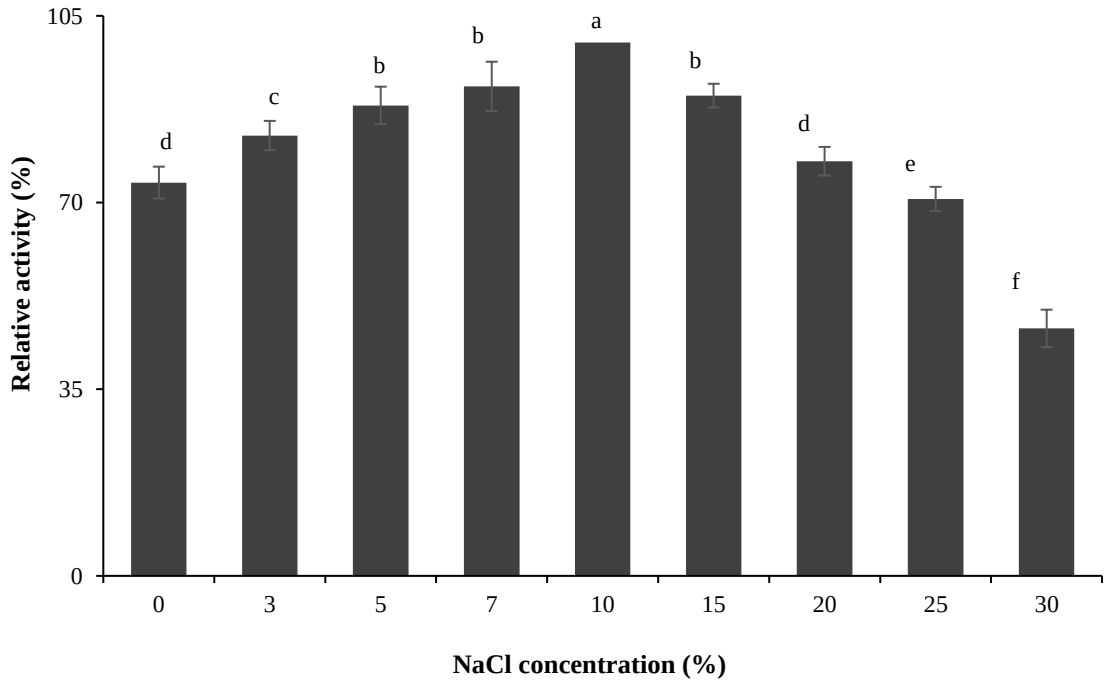



**Figure 22**. Stability of crude protease from *V. halodenitrificans* MSK-10P under various NaCl concentrations (%). The highest activity at optimum NaCl concentration was used as the standard. Error bars represent the standard deviations (n=3).

#### *4.3.1.5 Effect of inoculum size on protease production*

Effect of inoculum size on protease production was also studied in this experiment. The increasing of inoculum size from 0.5-2.5% showed increasing in enzyme production reaching the optimum at 2.5% level (27.37 U/mL) while further increasing the inoculum size caused reducing the protease production (Figure 23). Similar reports on the maximum protease production under 3% inoculum size were observed by other researchers. Zambare *et al*. (2011) and Nilegaonkar *et al*. (2007) reported maximum protease production under 3% inoculum size by *P. aeruginosa* and *B. cereus*. High inoculum size showed reduction of protease production. Lack of oxygen and reduction of nutrient could be the problem if high inoculum size was applied (Malherbe *et al*., 2007). This was confirmed by Haritha *et al*. (2012) who also observed the reduction in protease production by 36.3 and 58.4% in the inoculum size at 8.0 and 10.0%, respectively.

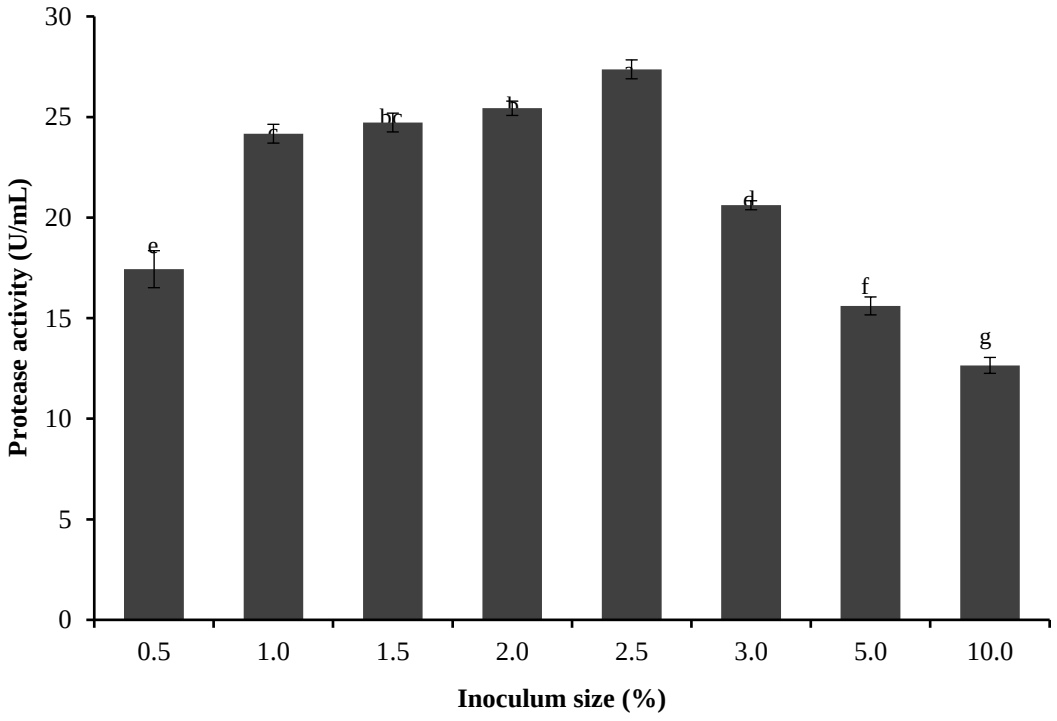



**Figure 23**. Effect of the inoculum size for protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in modified PM under shaking at 150 rpm for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

### *4.3.1.6 Effect of agitation speed on protease production*

The suitable agitation speed could assist in the mixing process of the available nutrients in the medium. Agitation also plays a significant role in the microbial growth. The suitable speed of agitation will support the success of the fermentation process (Ibrahim *et al*., 2015). In this experiment, the agitation at 150 rpm gave highest protease production with activity of 27.24 U/mL (Figure 24). The lowest production was shown at agitation speed of 100 rpm which could probably caused by lack of oxygen and nutrients needed by the bacteria. Low production also showed at higher agitation speed which could probably because of the highest speed may contribute to the damage of the cell structure. Agitation not only increases the transfer of mass and oxygen between distinct phases but also sustains homogeneous conditions of the system through a continuous mixing process. In addition, shear forces could possibly happen, which will affect the state of microorganisms, such as morphological changes, various growth performance, development of metabolite, and even cause cell destruction (Kim *et al*., 2003; Zhou *et al*., 2018).

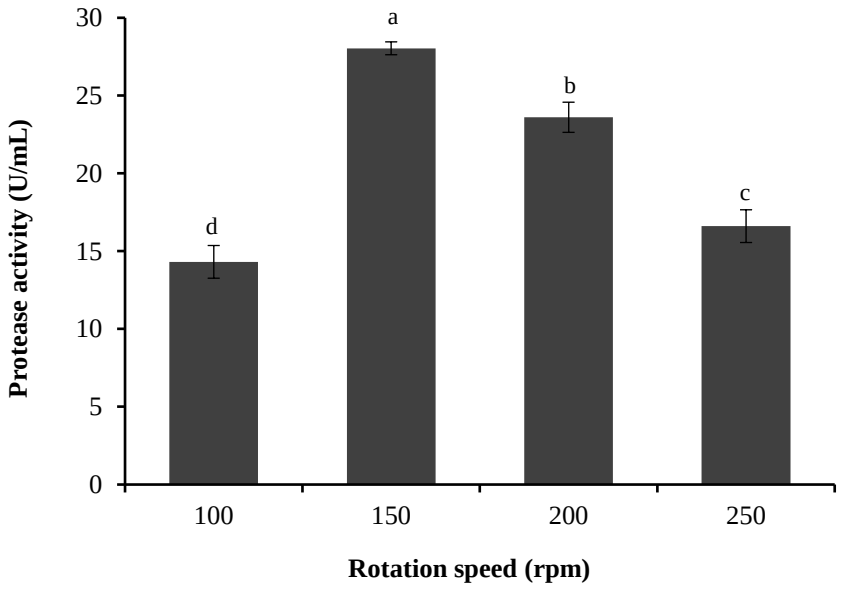



**Figure 24**. Effect of the agitation speed on protease production by *V. halodenitrificans* MSK-10P. Cultivations was performed in modified PM at 37ºC for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

### 4.3.2 Time course production of protease by *Virgibcillus* MSK-10P

Figure 25 shows the time course of protease production under the optimized conditions obtained from previous optimization process for 72 h. The protease activity was observed to increase steadily to the highest value at 36 h, and thereafter a steady decline in activity until 72 h. The activity at 36 h (36.32 U/mL) showed 3.83-fold higher than the initial activity before optimization process (9.47 U/mL). Cultivation time for protease production and its activity may differ among different organisms. As an example, the highest activity of protease produced by *Virgibacillus* sp. EMB13 was obtained at 60 h of cultivation. Other report for protease production by *B. licheniformis* isolated from saltern sediments found the maximum protease activity was obtained at 24 h (Suganthi *et al*., 2013). Meanwhile, cultivation time at 72 h gave the highest protease activity of *Virgibacillus* sp. TKNR13-3 (Tanasupawat *et al*., 2011). Several factors influence protease activity, thus, affect its production. Cultivation time may impact the production of protease. In addition, differences in medium pH, cultivation temperature, and salt concentration, may contribute also in protease activity and production (Pant *et al*., 2015). In addition, Figure 25 shows that the protease production followed the growth-associated model in which the protease was produced along with the growth of the microbial cells and the product concentration is almost directly proportional to microbial growth. However, the protease production was sharply decreased from 48 h while the microbial growth showed constant. This could probably cause by the production of other metabolites during the stationary phase and the competition of uptaking the substrate by the cells for its growth and for the protease production (Mazzuti *et al*., 2007; Wortel *et al*., 2018).

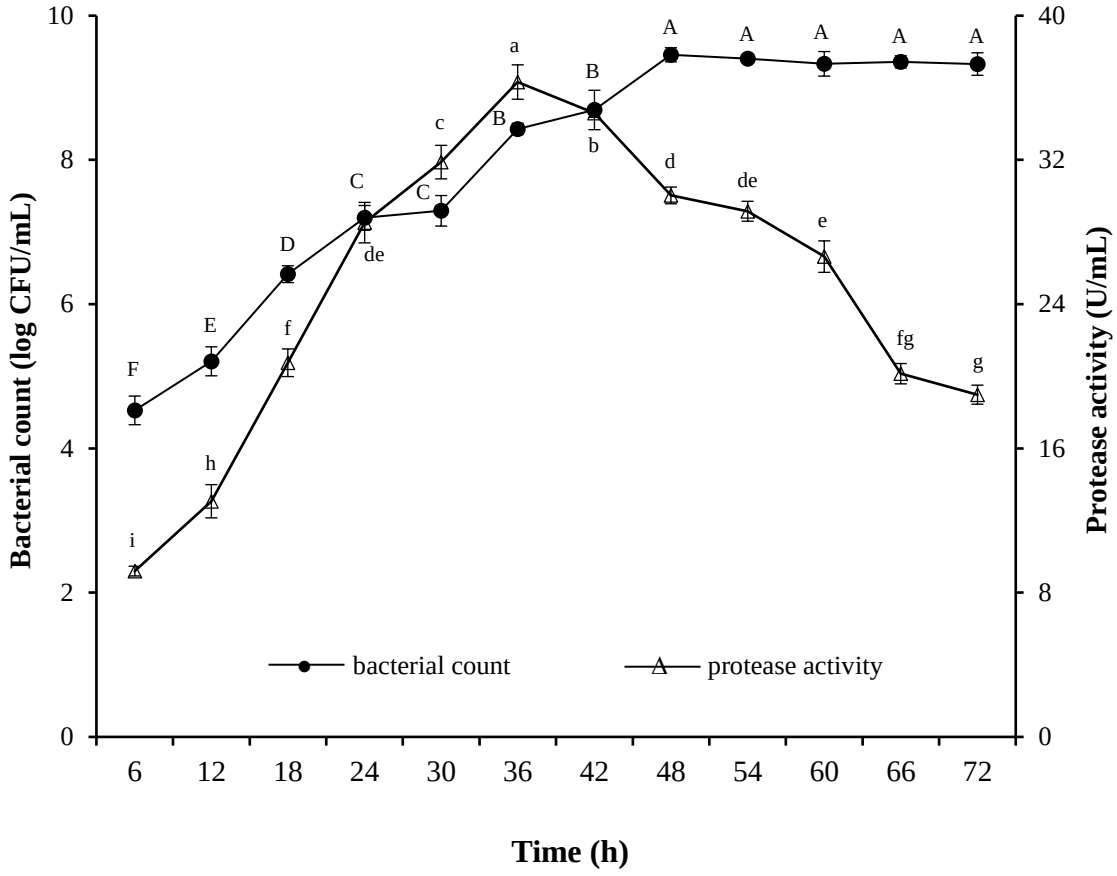



**Figure 25**. Time-course of protease production by *V. halodenitrificans* MSK-10P at selected optimum conditions (temperature 37ºC, pH 8.0, 1% (w/v) casein, 10% (w/v) NaCl, 2.5% (v/v) inoculum, and under agitation at 150 rpm). Error bars represent the standard deviations (n=3). Different uppercase and lowercase letters indicate significant differences ($p$<0.05).

## 4.4 Conclusions

In this study, the cultivation conditions affecting protease production by *V. halodenitrificans* MSK-10P has been optimized by using OFAT method. The highest protease production was obtained after 36 h of cultivation in the production medium contained 1.0% casein (w/v), 10% NaCl (w/v), 2.5% (v/v) inoculum size, and pH 8.0, under growth condition of temperature at 37ºC and agitation 150 rpm. The highest protease production at this optimum condition was 36.32 U/mL showing 3.83-fold

increase from the initial production of 9.47 U/mL. Based on the time-course experiment, this protease showed the growth-associated enzyme model. In addition, the stability of crude protease of *V. halodenitrificans* MSK-10P toward various NaCl concentration showed that it can be classified as halo-tolerant protease since it was stable at a broad range of NaCl concentration (0-25% w/v). Therefore, this protease has the potential to be applied in the industrial process under saline environment.

# CHAPTER 5

# OPTIMIZATION OF LIPASE PRODUCTION BY *Staphylococcus xylosus* LSM16


## Abstract

An optimization study was conducted for determining several optimum parameters contributed in lipase production by *Staphylococcus xylosus* LSM16, a strain isolated from fermented shrimp paste (Kapi). This strain was selected because it exhibited high lipase activity as well as the technological properties compared with other four isolated lipase-producing bacteria previously isolated. A one-factor-at a-time (OFAT) method has been conducted in order to select the suitable parameter affecting its lipase production. The temperature and initial pH optimum for lipase production were 37 $^{o}$C and 7.0. In addition, 2.0% (w/v) olive oil, 10% (w/v) NaCl, and 1.5% (v/v) inoculum concentration were all gave the highest lipase production as well as the agitation speed at 150 rpm. Meanwhile, time course experiment using the selected optimum conditions showed that at incubation time 48 h the lipase production was the highest (2.27 U/mL) which indicated 3.34-fold from initial production (0.67 U/mL). Furthermore, this lipase also showed halotolerance since the lipase activity was stable at wider range of NaCl concentration (0–20%, w/v).


Keywords: Kapi, lipase, *Staphylococcus xylosus* LSM16, optimization.

## 5.1 Introduction

Lipases (EC 3.1.1.3) are groups of hydrolytic enzymes which facilitate the breakdown of ester bonds in long-chain acylglycerols through hydrolysis reaction. Lipases contributed to high industrial enzyme market since it is important in biotechnological process (Sangeetha *et al*., 2011). Lipases are commonly used in a broad range of applications, such as in the fine chemicals' synthesis process; fats, oils, and food processing; cosmetics and pharmaceutical production; textile and paper manufacturing; wastewater treatment; the detergent and degreasing agents formulations; as well as in biodiesel production (Bayoumi *et al*., 2007). Lipases can be obtained from various sources, from animals to microorganisms. Microbial lipases considered as more useful source of lipase because of their high yields, various catalytic activities, not dependent of seasonal aspect, and fast growth on cheaper media. In addition, microbial lipases are also more stable comparing with plant and animal lipases (Verma *et al*., 2008).

Several parameters influence lipase production including nutritional, chemical, and physical conditions such as the carbon and nitrogen sources, temperature, pH of the medium, type of inducer, and agitation. The presence of certain inhibitors, activators, the quantity and origin of inoculum as well, can also influence the production of lipases (Ebrahimpour *et al*., 2008).

Staphylococci lipases has been known for their high contribution for developing aroma in fermented food products (Talon *et al*., 1997). Numbers of *Staphylococcus* species have been found as lipase sources for industrial purposes such as lipase from *Staph. arlettae* (Chauhan *et al*., 2013), *Staph. aureus* (Horchani *et al*., 2009; Hu *et al*., 2012), *Staph. epidermidis* (Chang *et al*., 2000), *Staph. haemolyticus* (Lee and Kim, 2015), *Staph. lipolyticus* (Arora, 2013), *Staph. simulans* (Sayari *et al*., 2001); *Staph. warneri* (Yokoi *et al*., 2012), and *Staph. xylosus* (Bertoldo *et al*., 2011; Bouaziz *et al*., 2011). Staphylococcal lipases are mostly extracellular and like other lipases, their production influenced by several nutritional, chemical, and physical factors (Gotz *et al*., 1998; Horchani *et al*., 2012).

*Staph. xylosus* is widely applied as lipolytic starter culture in the dairy and meat fermentation such as sausages, ham, milk, and cheese. Its lipase activity played a significant role in the fermentation process (Talon *et al*., 1996; Ghribi *et al*., 2009). In this study, a salt-tolerant lipase-producing bacterium, *Staph. xylosus* LSM16, which was previously isolated from Thai fermented shrimp paste (Kapi) has been studied for its lipase production. This strain was selected not only because it has the highest lipase activity compared with other strains but this strain also has various technological properties and safety attributes, such as antimicrobial activity toward several pathogenic bacteria, γ-hemolysis, weak ability to produce biofilm, susceptible to several tested antibiotics, and neither carry any enterotoxin genes nor produce biogenic amines. Various nutritional, chemical, and physical parameters contributed to lipase production were optimized by applying "one-factor-at a-time" (OFAT) method to obtain high lipase production.

## 5.2 Materials and Methods

### 5.2.1 Bacterial strain and inoculum preparation

*Staph. xylosus* LSM16 was selected to be optimized for its lipase production. A loopful of *Staph. xylosus* LSM16 strain after growth on TSA plate at 37°C for 24 h was inoculated in a flask containing 25 mL of tryptone soya broth (TSB, Himedia, Mumbai, India) medium supplemented with NaCl 10% (w/v). The flask was incubated at 37°C under agitation at 150 rpm for 24 h. The cell culture was used as inoculum when its optical density at 600nm (OD600) was 1.0. Then, 1% (v/v) of inoculum was transferred into lipase production medium. Lipase production medium (LPM) consisted of TSB medium supplemented with (% w/v): olive oil 1.0, gum Arabic 0.1, and NaCl 10.0, with pH set at 7.0. Culture broth of *Staph. xylosus* LSM16 was harvested by centrifugation (9800 × *g*) at 4$^{o}$C for 10 min and used as crude lipase for determination of lipase activity.

### 5.2.2 Optimization of lipase production by *Staph. xylosus* LSM16

The one-factor-at a-time (OFAT) experiment was used to determine the optimum condition for lipase production. Initial conditions were as follows: pH medium was 7.0, time of incubation was 24 h, and agitation speed was 150 rpm.

#### *5.2.2.1 Effect of temperature on lipase production*

The optimum temperature for lipase production was studied by culturing *Staph. xylosus* LSM16 in TSB containing 10% (w/v) NaCl. The incubation was conducted at various temperatures (25-50°C) and shaking at 150 rpm for 24 h. After incubation, the lipase production was determined in terms of lipase activity by using copper-soap method as previously described in Chapter 3.

#### *5.2.2.2 Effect of initial pH medium on lipase production*

The initial pH optimum on lipase production was investigated by culturing the strain in LPM at various pH: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. The pH of the medium was adjusted using 0.1N HCl or 0.1N NaOH. The cultivation was performed at selected optimum temperature from section 5.2.2.1 for 24 h with constant agitation speed at 150 rpm. The lipase production was determined in terms of activity.

#### *5.2.2.3 Effect of oil on lipase production*

The effect of different type of oil on lipase production was investigated with several oils, such as olive oil, palm oil, sunflower oil, rice bran oil, sesame oil, soybean oil, and corn oil (in 1.0%, w/v). Furthermore, the effect of different concentrations of selected oil (0.5, 1.0, 1.5, 2.0, and 2.5% w/v) was also investigated. The cultivation was performed with the optimum temperature and pH as selected from section 5.2.2.1 and 5.2.2.2 under shaking speed at 150 rpm for 24 h. Afterwards, the lipase production was determined in terms of activity.

#### *5.2.2.4 Effect of NaCl concentration on lipase production*

The effect of various NaCl concentrations on lipase production was investigated. Various NaCl concentrations (%, w/v) added into LPM were: 0, 3, 5, 10, 15, 20, and 25. The lipase activity was determined after incubation for 24 h at the selected conditions previously obtained with shaking speed at 150 rpm.

In addition, the stability of lipase under various NaCl concentrations was also investigated. Crude lipase was pre-incubated in phosphate buffer (50 mM, pH 7.0) containing various NaCl concentrations (% w/v) of 0, 3, 5, 10, 15, 20, 25, and 30, for 2 h at 37$^{o}$C. The relative activity of crude enzyme was determined using the maximum activity of the respective NaCl concentration as 100%.

#### *5.2.2.5 Effect of inoculum concentration on lipase production*

The effect of the inoculum concentrations on lipase production was studied. Various concentrations of bacterial culture ((% v/v): 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, and 10.0) were added to the medium with the selected optimum NaCl concentration. The lipase production was determined after incubation for 24 h under previously selected conditions.

#### *5.2.2.6 Effect of agitation speed on lipase production*

The optimum agitation required for lipase production was investigated at various shaking speeds (rpm): 100, 150, 200, and 250. The lipase production was determined in terms of activity after incubation for 24 h under previously selected conditions.

### 5.2.3 Time course production of lipase

For the time course study of the lipase production, the combination of selected conditions and medium from sub-section 5.2.2.1-5.2.2.6 was used to monitor the bacterial count and the protease production. The bacterial culture with optimal inoculum concentration was transferred into 50 mL of LPM containing suitable oil and concentration of NaCl with optimum initial pH. The culture was incubated at selected temperature and agitation for given period (120 h). The samples were taken

continuously at 12 h of the time interval until 120 h for measuring the cell growth and lipase production. The cell growth was measured by using spread-plate method on TSA plate supplemented with 10% (w/v) NaCl and 2% (w/w) olive oil.

### 5.2.4 Lipase activity assay

Lipase activity was determined according to copper-soap method developed by Lee and Rhee (2008) previously described in Chapter 3, sub section 3.1.2.

### 5.2.5 Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using SPSS v 16.0. Analysis of Variance (ANOVA) and mean comparison by Duncan's Multiple Range Test (DMRT) were analyzed.

## 5.3 Results and Discussion

### 5.3.1 Optimization of lipase production

#### *5.3.1.1 Effect of temperature on lipase production*

To determine the optimum temperature for the lipase production by *Staph. xylosus* LSM16, the experiment was conducted at temperature range 30-50$^{o}$C (Figure 26). The results showed that incubation temperature significantly affected the lipase production by *Staph. xylosus* LSM16. At room temperature around 30$^{o}$C, the lipase activity was 0.60 U/mL. The optimum temperature was 37$^{o}$C (0.68 U/mL) showing activity slightly higher than the incubation temperature at 40$^{o}$C (0.64 U/mL, $p$<0.05). Lipase production decreased significantly at higher temperature, 45 and 50$^{o}$C (0.41 and 0.33 U/mL, respectively). This result was in accordance with Ghribi *et al*. (2009) who found that the optimum temperature for lipase production by *Staph. xylosus* was 37$^{o}$C (42 U/mL), which was determined titrimetrically. Different results by other researchers found that the temperature optimum for the production of *Staph. xylosus* lipase were 45 and 55$^{o}$C (Brod *et al*., 2010; Bouaziz *et al*., 2011). The range of optimum temperature by most of bacterial lipase was reported at 30-60$^{o}$C (Gupta *et al*., 2002), while the optimum temperature for staphylococcal lipases was observed around 30-55$^{o}$C (Horchani *et al*., 2012).

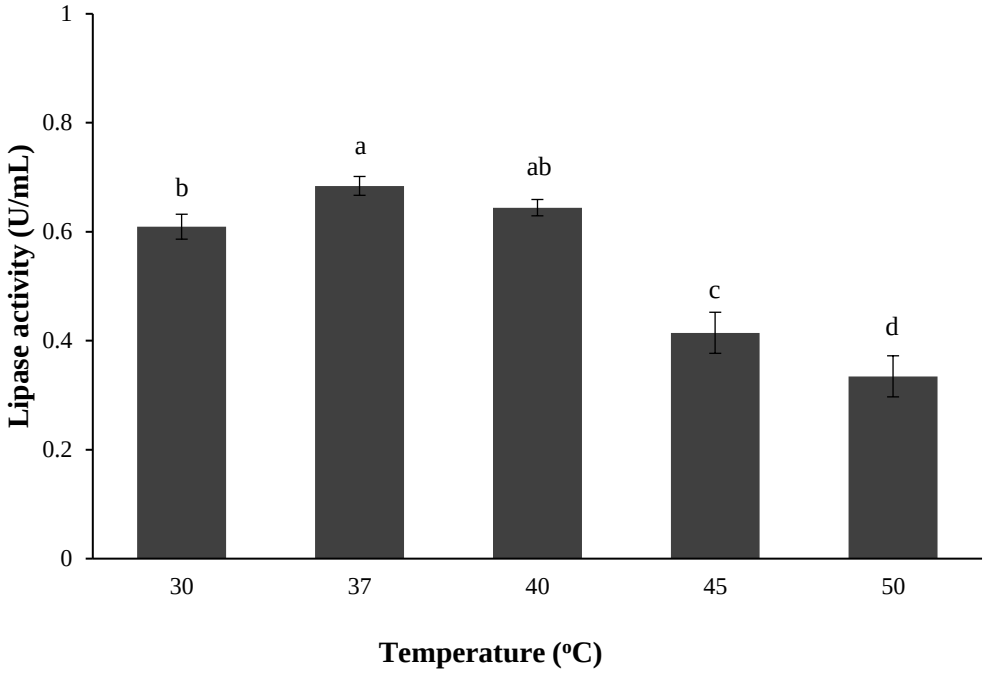



**Figure 26**. Effect of the incubation temperature on lipase production by *Staph. xylosus* LSM16. Cultivation was performed in LPM (pH 7.0) under shaking at 150 rpm for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

As it is widely known that each of bacterium has different optimum temperature for lipase production. Mostly bacteria have optimum temperature around 30-37°C. Cultivation at lower or higher than optimum temperature may cause decreasing of lipase production. It is because at higher temperature the enzyme can be inactivated, while at lower temperature may inhibit the nutrients flow through the cell membrane which may lead the decrease of enzyme production (Aiba *et al*., 1973; Vieille and Zeykus, 2001).

#### *5.3.1.2 Effect of initial pH on lipase production*

The effect of pH on lipase production by *S. xylosus* LSM16 was investigated. Figure 27 shows that the lipase production significantly increased from pH 4 to 7 and decreased sharply from pH 8-11. It was found that the optimum initial

pH was 7.0 with the activity of 0.69 U/mL. Other reports reported that pH optimum for lipase production by *Staph. xylosus* were 8.5 and 9.0 (Bouaziz *et al*., 2011; Brod *et al*., 2009), while Horchani *et al.* (2012) reported that the optimum pH for lipase production by staphylococci was vary from 6.0 to 9.5.

pH is known to affect the synthesis and secretion of enzymes. The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the microorganism thus lowering their overall metabolic activity including enzyme production (Willey *et al*., 2008).

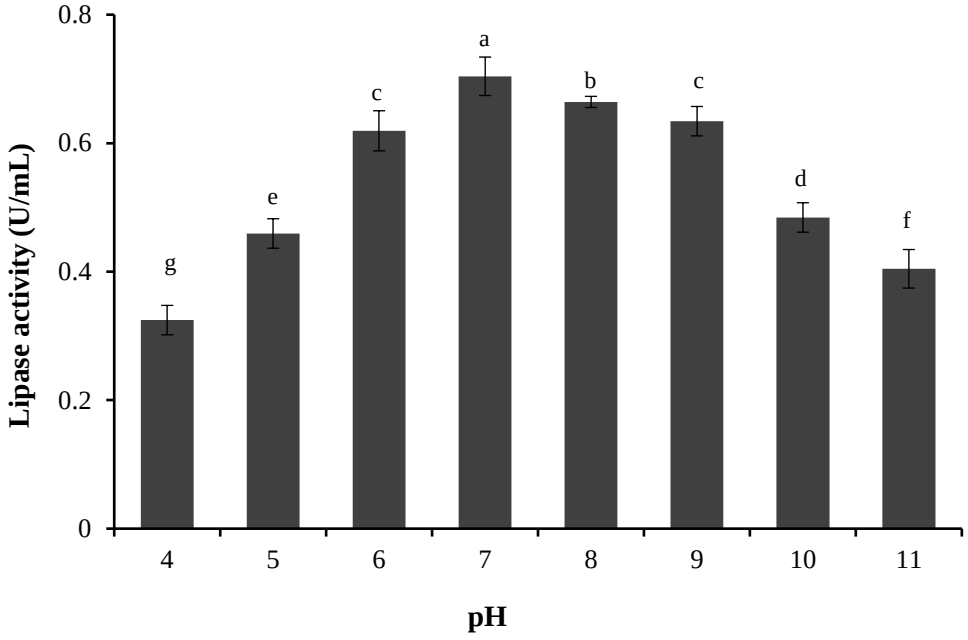



**Figure 27**. Effect of the initial pH on lipase production by *Staph. xylosus* LSM16. Cultivation was performed in LPM under shaking (150 rpm) at 37ºC for 24 h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

#### *5.3.1.3 Effect of oil on lipase production*

The effect of various type of oils on lipase production including olive oil, palm oil, rice bran oil, sunflower oil, corn oil, soybean oil, and sesame oil were

investigated. The highest lipase activity was detected in LPM containing olive oil (Figure 28). Similar reports were obtained by Bouaziz *et al*. (2011) and Khoramnia *et al*. (2010) who found that olive oil was the suitable carbon source for lipase production by *Staph. xylosus*. It can be seen from Figure 29 that olive oil at 2% (w/v) gave the highest lipase production compared with other concentrations of olive oil. The lipase production with 2% (w/v) olive oil (1.14 U/mL) showed 1.7-fold higher than initial production before optimization (0.67 U/mL). It has been reported that olive oil contains 55-83% oleic acids (Boskou *et al*., 2006). Olive oil contains the highest oleic acid compared with other tested oils. Darvishi *et al*. (2009) found that oleic acid was the most influence compound to the production of lipase.

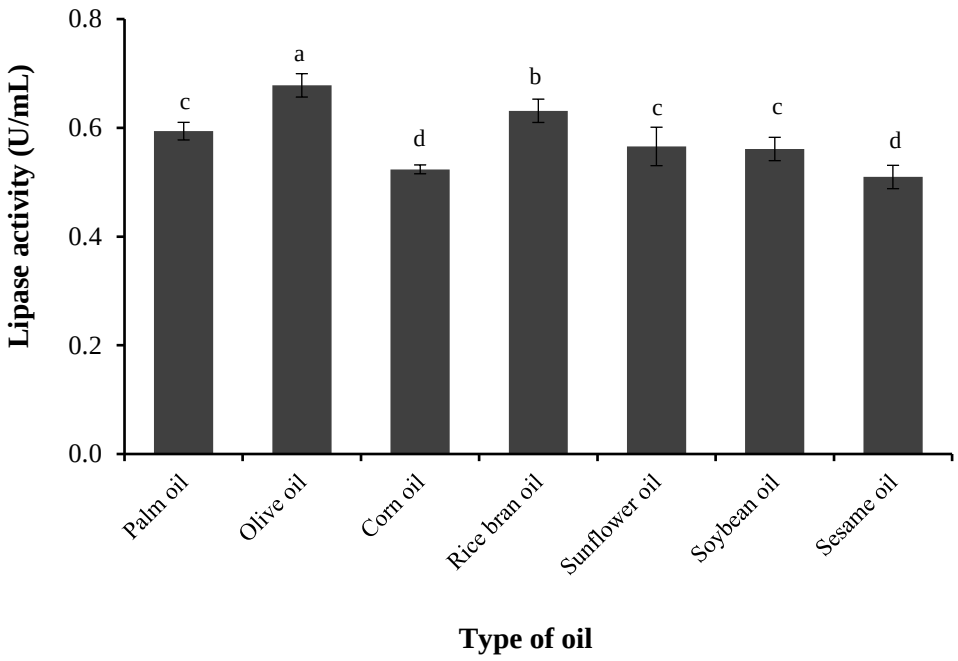



**Figure 28.** Effect of oil on lipase production by *Staph. xylosus* LSM16. Cultivation was performed in LPM (pH 8.0) at 37$^{o}$C for 24h under shaking at 150 rpm. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

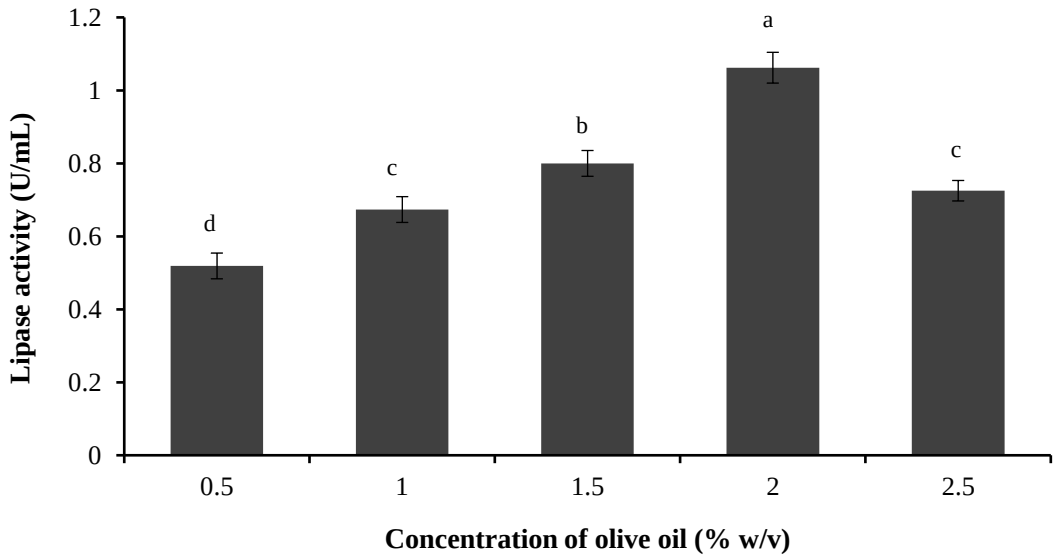



**Figure 29**. Effect of the concentration of olive oil (% w/v) on lipase production by *Staph xylosus* LSM16. Cultivation was performed in LPM (pH 7.0) at 37ºC for 24h with shaking at 150 rpm. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

#### *5.3.1.4 Effect of NaCl concentration on lipase production*

The effect of various NaCl concentrations on lipase production by *S. xylosus* LSM16 was investigated. It can be seen from Figure 30 that lipase production increased with increasing the NaCl concentration (0–7 % NaCl, w/v) and reached its maximum at 10 % NaCl. However, NaCl higher than 10% caused decreasing of lipase production. At optimum NaCl concentration (10%), the production (1.07 U/mL) showed 1.6-fold higher than initial production (0.67 U/mL. The higher NaCl concentration away from optimum point the lower production obtained (Rao *et al*., 2009). It can be explained that increasing the concentration of salt means increasing the number of ions in solution which lead to unbalancing the electrostatic ions out of optimum concentration needed (Chowdhury, 2007). This imbalance may disrupt the metabolism process in bacteria for synthesizing proteins (Willey *et al*., 2008).

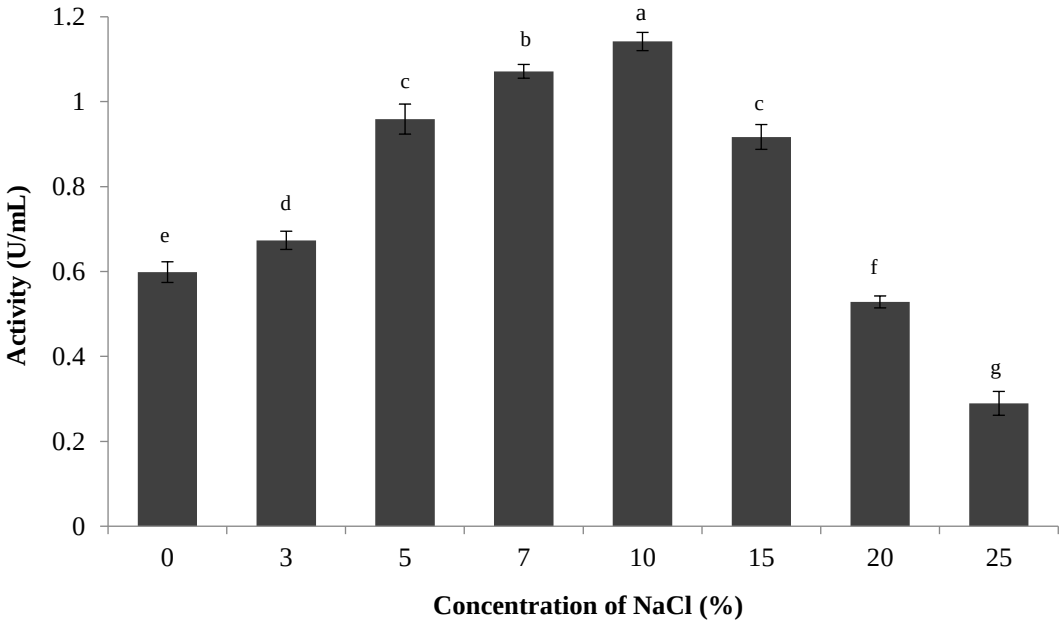



**Figure 30**. Effect of the NaCl concentration (% w/v) on lipase production by *Staph xylosus* LSM16. Cultivation was performed in LPM (pH 7.0) at 37ºC for 24h with shaking at 150 rpm. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

In this experiment, the lipase stability of *Staph. xylosus* LSM16 was also investigated at various NaCl concentration (0 – 30%, w/v). It can be shown in Figure 31 that lipase of *Staph. xylosus* LSM16 can retain its activity over the 0% NaCl (67.49%) until 20% NaCl (60.22%) compared with the highest activity at 10% NaCl. Meanwhile, at 25% and 30% NaCl, its activity drastically lost to only 23.87% and 18.73 %, respectively. It can be assumed that the lipase of *Staph. xylosus* LSM16 can be categorized as halotolerant lipase, since it can have the activity over a wide range of NaCl concentration, from 0% to 15% NaCl. The stability of an enzyme at several points of salt concentrations could also be an indication that the enzyme is stable in the environment of low water activity that usually arises in enzymatic reactions which take place in organic solvents (Eichler, 2001). In addition, because *Staph. xylosus* can also be categorized as halotolerant bacterium, then the synthesis amount and the activity in

catalysis of its enzymes can be moderately high in the medium without salt or even in high salinity (Chauhan *et al.*, 2013).

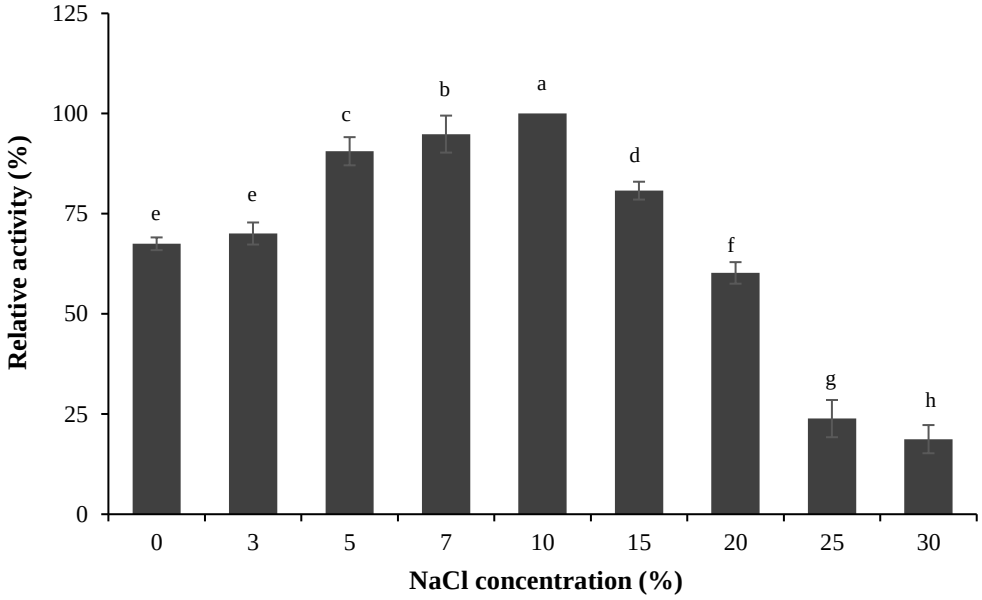



**Figure 31**. The effect of NaCl concentration on *Staph. xylosus* LSM16 lipase stability. All values are represented as ±SD (n=3).

#### *5.3.1.5 Effect of inoculum concentration on lipase production*

The effect of inoculum concentration on lipase production by *Staph. xylosus* LSM16 was investigated. From Figure 32, 1.5% (v/v) of 24-h inoculum gave the highest lipase production (1.31 U/mL). If comparing with the initial production in this experiment which was added with 1% (v/v) inoculum (0.67 U/mL), it can be figured out that the production was increased 1.96-fold. Meanwhile, at higher concentration of inoculum (2.0–10.0%), the lipase production decreased. If the amount of inoculum used is low, it will reduce the number of products. Whereas if the amount of inoculum is too much, it can produce too much biomass which causes the formation of a poor product (Mudgetti, 1996). Enzyme production would be optimum at optimal level of inoculum because at this point the equilibrium is maintained between the availability of inoculum and the substrates. Meanwhile, the decline in enzyme yield at higher inoculum size might be due to formation of thick suspensions and improper mixing of substrates in

shake flasks which lead to a depletion in oxygen and nutrients in the culture media (Abol-Fotouh *et al*., 2016). With subsequent increase in inoculum size resulted rapid depletion of macro and micronutrients and thus inhibits their growth and enzyme production (Omojasola *et al*., 2009).

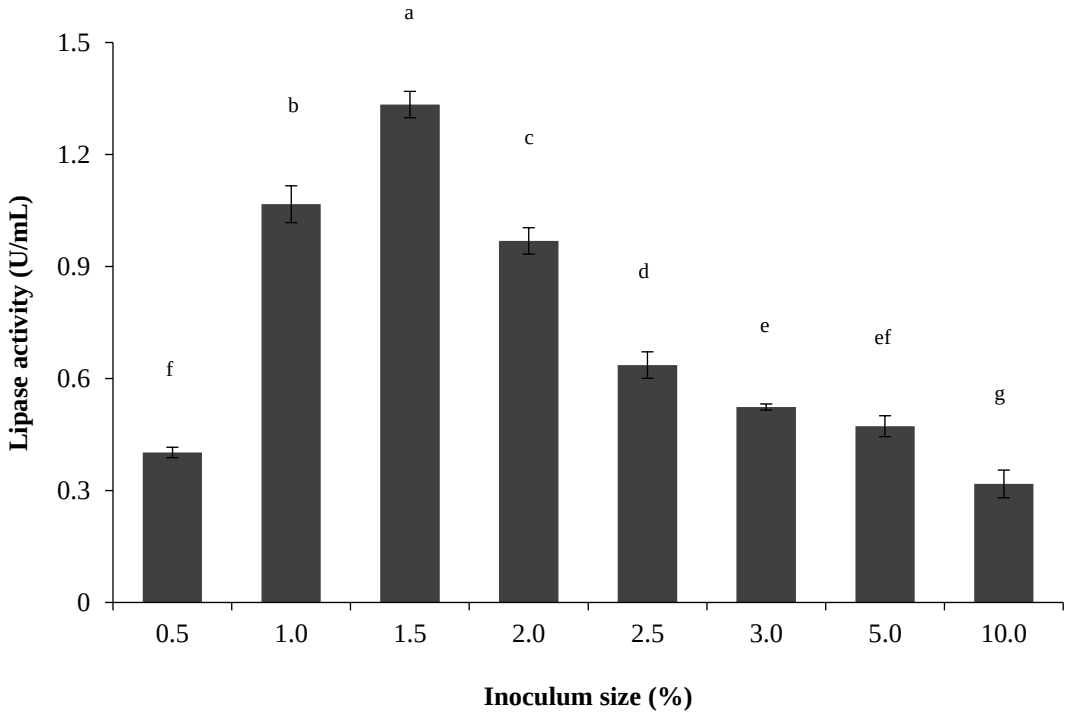



**Figure 32**. Effect of the inoculum size (% v/v) on lipase production by *Staph xylosus* LSM16. Cultivation was performed in LPM (pH 7.0) at 37ºC for 24h with shaking at 150 rpm. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

#### *5.3.1.6 Effect of agitation speed on lipase production*

The suitable agitation speed in lipase production by *Staph. xylosus* LSM16 was determined. It is depicted in Figure 33 that lipase production increased from 100 – 150 rpm, afterward the lipase production decreased at higher agitation speed. The lipase production showed the highest (1.35 U/mL) at 150 rpm which increasing 2.02-fold higher than the initial production (0.67 U/mL) before optimization.

Similar report was obtained for *Staph. epidermidis* lipase which also had optimum lipase production at 150 rpm (Edupaganti *et al*., 2017), while both lipase of *Staph. aureus* and *Staph. lipolyticus* had optimum production at 200 rpm (Horchani *et al*., 2009; Azora, 2013). The level of agitation not only affects the availability of oxygen in the medium but also influences the availability of other nutrients. A rotation speed of 100 rpm might contribute to limit the level of oxygen, which caused the lack of homogenized condition of the medium as well as the reduction of available lipid consumed by the cells. Consequently, lower lipase production levels would obtain (Alonso *et al*., 2005). Meanwhile, the reduction of enzyme production at higher levels of agitation can be associated with the effects of shear stress on bacterial cells which in turn could decrease the enzyme production (Sooch and Kauldar, 2013).

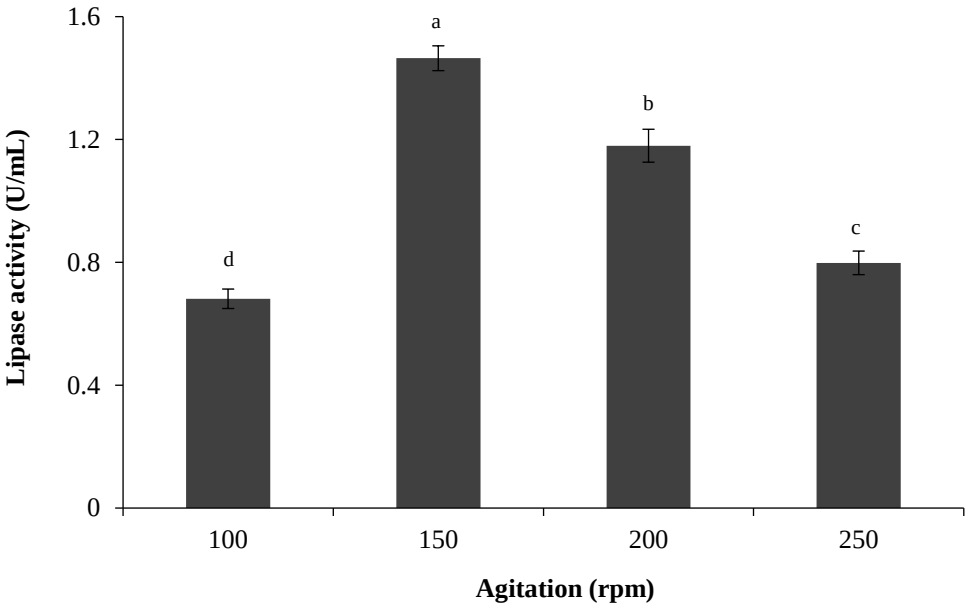



**Figure 33**. Effect of the agitation speed (rpm) on lipase production by *Staph xylosus* LSM16. Cultivations was performed in LPM (pH 7.0) at 37ºC for 24h. Error bars represent the standard deviations (n=3). Different lowercase letters indicate significant differences ($p$<0.05).

### 5.3.2 Time-course production of lipase by *Staph. xylosus* LSM16

The time-course productions of lipase by *Staph. xylosus* LSM16 was studied by cultivating the strain at the optimum condition. As previously obtained from section 5.3.1.1.-5.3.1.6., the optimum condition for lipase production by *Staph. xylosus* LSM16 was obtained by cultivating the strain in the medium at 37$^{o}$C, pH 7.0, and agitation at 150 rpm, using 2% (w/v) olive oil as substrate with 1.5% (v/v) inoculum and supplemented with 10% (w/v) NaCl. Low production of lipase (0.46 U/mL) was detected at the beginning of cultivation (0 – 12 h) whereas the bacterial cells grew well at this period. After that, the lipase production increased until 48 h of incubation while the bacterial cell grew until 60 h (Figure 34). The lipase production showed maximum at 48 h (2.27 U/mL) which demonstrated 3.34-fold increasing compared with initial production at 0.67 U/mL. The lipase production by *Staph. xylosus* LSM16 follows the growth-associated model, in which the lipase was produced along with the growth of the strain. While the strain reached the stationary phase at 60 h, the lipase production decreased. This might be due to exhaustion of nutrients, environmental changing in the medium, or proteolysis of the lipase by proteases which were might also be produced simultaneously (Nouroozi *et al*., 2005).

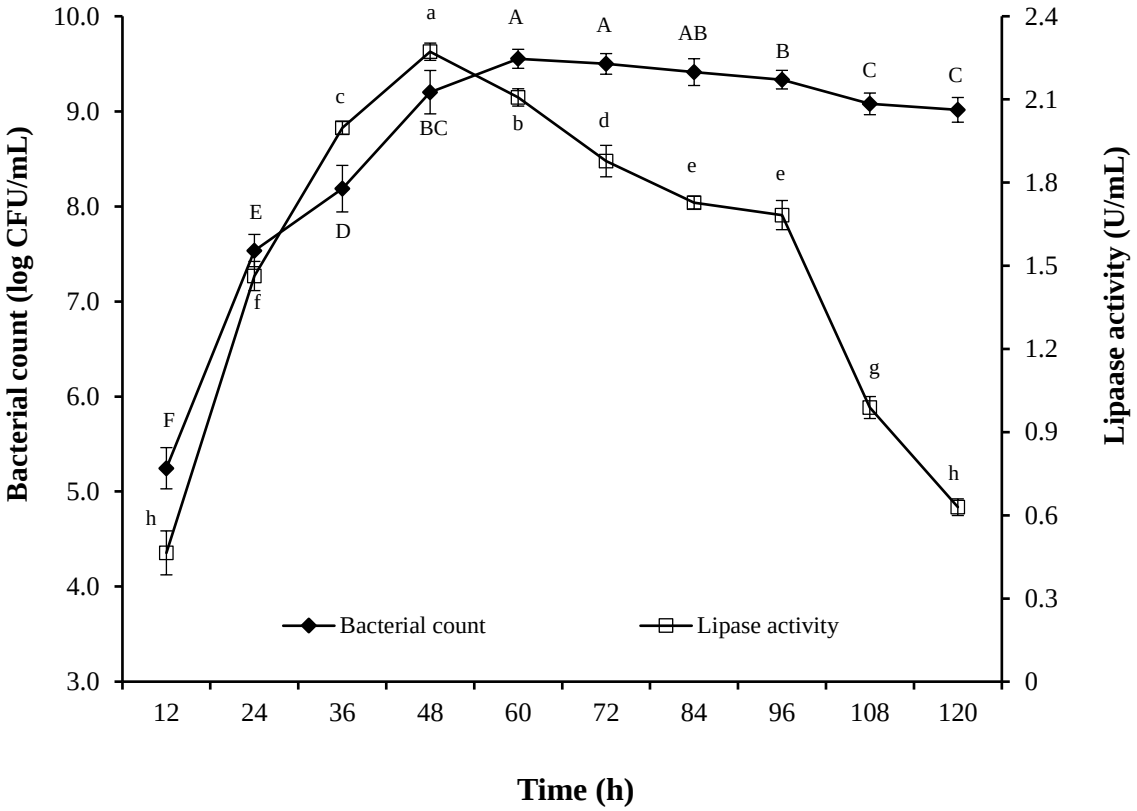



**Figure 34**. Time-course of lipase production by *Staph. xylosus* LSM16 at selected optimum conditions (temperature 37°C, pH 7.0, 2% (w/v) olive oil, 10% (w/v) NaCl, 1.5% (v/v) inoculum, and under agitation at 150 rpm). Error bars represent the standard deviations (n=3). Different uppercase and lowercase letters indicate significant differences ($p$<0.05).

## 5.4 Conclusions

In this study, OFAT method was applied to investigate the optimum cultivation conditions for lipase production by *Staph. xylosus* LSM16. The highest lipase production was obtained after 48 h of cultivation at 37°C in the production medium contained 2.0% olive oil (w/v), 10% NaCl (w/v), 2.0% (v/v) inoculum size, and pH 7.0, under agitation at 150 rpm. The highest lipase production at this optimum condition was 2.27 U/mL which showed 3.34-fold increase from the initial production

of 0.67 U/mL. Based on the time-course experiment, this lipase production was a growth-associated enzyme model. In addition, the stability of crude lipase of *Staph. xylosus* LSM16 toward various NaCl concentration showed that it can be classified as halo-tolerant lipase since it was stable at a broad range of NaCl concentration (0-15% w/v).

# CHAPTER 6

# APPLICATION OF SELECTED PROTEASE- AND LIPASE-PRODUCING BACTERIA IN FERMENTED SHRIMP PASTE (KAPI) PRODUCTION


## Abstract

This research aimed to study the application of protease-producing bacteria, *Virgibacillus halodenirificans* MSK-10P and lipase-producing bacteria, *Staphylococcus xylosus* LSM16, in the Kapi production. Four treatments of Kapi were studied: (i) natural fermentation (as control, KC); (ii) inoculated with *V. halodenitrificans* MSK-10P (KP); (iii) inoculated with *Staph. xylosus* LSM16 (KL); and (iv) inoculated with both selected protease and lipase producing strain (KM). Physicochemical characteristic and microbiological safety of the Kapi were investigated. All the Kapi products inoculated with starter cultures met the physicochemical characteristics and microbiological safety required by Thai Industrial Standard (TIS) 1205/2006. Moisture, salt, and total nitrogen content of Kapi products were in the range of 40.02-42.73%, 40.01-42.79%, and 6.33-7.82%, respectively. Food-borne pathogens (coliform bacteria, *Clostridium perfringens*, *Staphylococcus aureus*, and *Salmonella* Typhimurium) were not detected in all finished Kapi product. Moreover, sensory analysis also showed that all Kapi inoculated with starter cultures had the score of overall acceptance by panelists higher than the spontaneous fermented Kapi and commercial product. These results suggesting that *V. halodenitrificans* MSK-10P and *Staph. xylosus* LSM16 could be applied as promising starter cultures in the production of Kapi.


Keywords: Kapi, starter culture, *Virgibacillus halodenirificans* MSK-10P, *Staphylococcus xylosus* LSM16.

## 6.1 Introduction

Starter culture application through traditional fermentation would improve the fermentation processes and the product quality. This could be accomplished by accelerating metabolic activities rapidly as well as providing desired sensory features. In addition, by applying starter culture the fermentation process could be more predictable and monitored. Moreover, the reduction of toxicological threats and the development of product safety could be generated (Holzapfel, 1997).

Application of starter culture to assists the seafood fermentation has been reported. Mainly, starter cultures exhibited the protease activity were used to reduce the length of seafood fermentation. As reported by Yongsawatdigul *et al.* (2007), *Virgibacillus* sp. SK 33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5 having proteinase activity has been applied as starter cultures in the production of fish sauce. Those bacteria also increased the free amino acids content as well as the necessary volatile compounds. In addition, Udomsil *et al.* (2011) reported that application of *Tetragenococcus halophilus*, a protease-producing lactic acid bacterium isolated from fish sauce fermentation can reduce the length of fish sauce fermentation as well as the content of BAs in fish sauce.

Lipase-producing bacteria has been extensively applied in the meat fermentation. Some coagulase-negative staphylococci (CNS) exhibited lipolytic activity has been widely reported as starter cultures in the sausage fermentation. *Staphylococcus carnosus* and *Staph. xylosus* are two main lipolytic-CNS applied. CNS play a significant role in the development of aroma as well as flavor and color of the fermented meat products (Talon *et al*., 1996; Essid *et al.*, 2007).

Kapi is a Thai traditional fermented shrimp paste which is mainly consumed as a condiment in many cuisines by Thai people. It is mainly produced from the tiny shrimp or krill (*Acetes* or *Mesopodopsis* species) and mixed with 10-30% (w/w) NaCl and be fermented for several months (Faithong and Benjakul, 2012). The addition of salt is to prevent food deteriorating, food poisoning, and generating fishy- and meaty-savory flavor as well (Steinkraus, 2002). Moreover, indigenous proteases- and lipases-producing microorganisms play significant role in mediating the enzymatic fermentation of shrimp which produce short chain peptides and free amino acids

contributed to typical flavor and umami taste (Yoshida, 1998). Therefore, Kapi is also a useful source of peptides and amino acids (Faithong *et al.*, 2010).

Many factors contributed to the quality of Kapi. The species of shrimp applied, the quantity of salt added, the treatment of raw materials prior to fermentation, and the length of fermentation time are the main factors giving impact to Kapi quality (Prapasuwannakul and Suwannahong, 2015).

Until now, very few reports are available for the application of starter cultures in Kapi fermentation. Only Pongsetkul *et al.* (2018) reported to apply *Bacillus* sp. KC3 as starter culture in the Kapi production. However, no report about safety assessment of the starter as well as the safety of the product. In Thailand, Kapi was mostly produced using spontaneous fermentation. Recently, we isolated and selected one strain of potential proteinase-producing bacteria and one strain of potential lipase-producing bacteria from Kapi. They were identified as *V. halodenitrificans* MSK-10P and *Staph. xylosus* LSM16 based on their morphological and some physiological characteristics, and their 16S rDNA gene sequences. Moreover, the safety attributes of these strains have also been checked. Therefore, the aims of the present study were to apply both strains as starter cultures in the production of Kapi to shorten the production time compared with the production by local producer as well as to characterize the physicochemical properties, microbiological quality, and consumer acceptance of the obtained Kapi products.

## 6.2 Material and Methods

### 6.2.1 Preparation of sample

For each batch of fermentation, 5 kilograms of tiny shrimp *Acetes* sp. were caught by the local fisherman from the coast in The-Pha District, Songkhla Province, Thailand. The production process was based on the local producer's recipe (Figure 35). The shrimps were washed to remove unwanted objects. Subsequently, the cleaned shrimps were mixed with 20% of sea salt. The mixtures then were dried under the sunlight for 6-8 h and starter cultures were applied. Afterwards, the mixtures were left overnight. Subsequently, the mixtures were dried under sunlight for 2-3 days. When

the mixture reached 70-80 % of dryness, they were grinded and kept in sealed containers about 4 weeks.

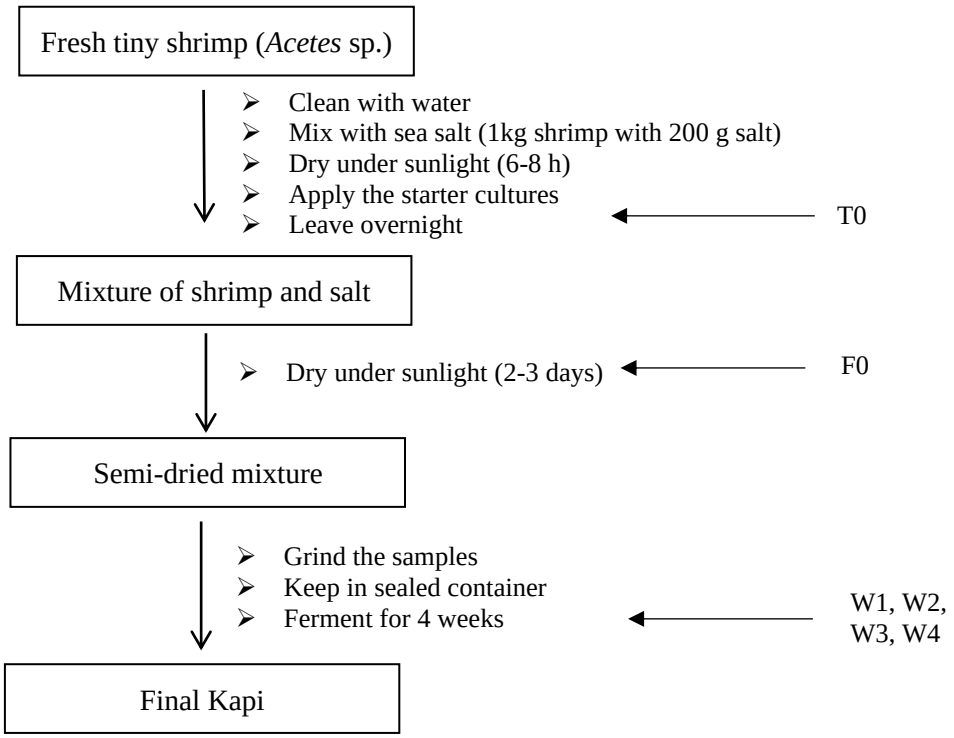



**Figure 35.** Scheme of Kapi production (time of sampling described as: T0: the day at starter cultures were applied; F0: after 3 days sundried; W1-W4: the first until fourth week of fermentation)

### 6.2.2 Production of Kapi

#### *6.2.2.1 Preparation of starter cultures*

For the preparation of starter cultures, 100 μL of the protease-producing *V. halodenitrificans* MSK-10P was inoculated in 10 mL of nutrient broth (NB, Himedia, Mumbai, India) supplemented with 10% NaCl (w/v) and incubated at 37$^{o}$C overnight. Two-mililiter of the active culture was transferred to 50 mL of NB and incubated at 37$^{o}$C for 24 h. Cells were harvested by centrifugation at 8000 × g for 15 min at 4$^{o}$C and washed twice with sterile saline water (0.85%). The cells pellet was resuspended in 50 mL of sterile saline and then applied as proteolytic-strain starter culture. In addition, 100 μL of the lipase-producing *Staph. xylosus* LSM16 was inoculated in 10 mL of tryptone soya broth (TSB, Himedia, Mumbai, India) supplemented with 5% NaCl (w/v)

and incubated at 37$^{o}$C overnight. Two-milliliter of the active culture was transferred to 50 mL of NB and incubated at 37$^{o}$C for 24 h. Cells were harvested by centrifugation at 8000 × g for 15 min at 4$^{o}$C and washed twice with sterile saline water (0.85%). The cells pellet was resuspended in 50 mL of sterile saline and then applied as lipolytic-strain starter culture.

#### *6.2.2.2 Fermentation of* Kapi

In order to study the effect of protease- and lipase-producing starter cultures on Kapi product, Kapi was produced in different 4 conditions: (i) spontaneous fermentation (as control, KC); (ii) inoculated with protease-producing *V. halodenitrificans* MSK-10P (KP); (iii) inoculated with lipase-producing *Staph. xylosus* LSM16 (KL); and (iv) inoculated with both selected protease- and lipase- producing strains (KM). The fermentation was conducted for 1. Kapi was sampling at the day of starter addition (T0), at the beginning of fermentation after 3 days sundried (F0) and every week during fermentation (W1, W2, W3, and W4), for measurement of the physicochemical properties and microbiological quality (Figure 35).

### 6.2.3 Physicochemical properties and microbiological quality of Kapi

#### *6.2.3.1 Physicochemical properties*

Some physicochemical properties such as moisture content, pH, salt content, and water activity (Aw) were determined and monitored during the fermentation process. Moisture content was determined by oven method (AOAC, 1990). The pH was measured by using a pH meter (STARTER2100, OHAUS, New Jersey, USA). The salt content was determined by gravimetry (Mohr method, AOAC, 2005) as follows: 25 mL of aqueous extract of each sample was titrated against 0.1 M $AgNO_3$ using 5% $K_2CrO_4$ as an indicator. Salt content was reported as percentage of NaCl on the basis of dry weight (the salt content obtained in wet basis divided by dry weight of samples). Meanwhile, the $A_w$ was measured at 25$^{o}$C using a water activity meter (Aqua Lab, Meter Group, Pullman, USA).

The color of final Kapi products from this study and commercial product (CP) was compared and determined by a colorimeter (Color Flex, Hunter Lab Reston,

Reston, VA, USA). Color components including lightness, redness/greenness, yellowness/blueness, and total difference of color, represented by L*, a*, b*, and ΔE*, respectively, were recorded. The ΔE* was calculated by the following formula:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Where ΔL*, Δa*, Δb* are the differences between color parameters of the samples and those of the standards (L* = 92.82, a* = -1.24, b* = 0.46).

Texture analysis of Kapi samples was compared and investigated using a texture analyzer (TA-XT Plus Analyzer, Stable Microsystems, Surrey, United Kingdom), with a 50 mm cylinder probe based. The parameters of analyzed textures were hardness, adhesiveness, springiness, cohesiveness, gumminess, and chewiness.

#### *6.2.3.2 Microbiological quality*

Microbiological quality of Kapi were monitored through fermentation process. Briefly, 25 g of sample was aseptically collected and put in a stomacher bag, and subsequently sterile 225 mL of 0.1% peptone water was added. The mixture was then mixed for 1 min in a stomacher (Seward 400 circulator, England). Afterwards, 10-fold serial dilution was prepared from the homogenate using the same 0.1% peptone water. Then, 100 μL of diluted samples were spread on certain appropriate growth media. Total viable count and Enterobacteriaceae were enumerated on plate count agar (PCA; Himedia, Mumbai, India) and violet red bile (lactose) agar (VRBA; Merck, Darmstadt, Germany) plates, respectively. Lactic acid bacteria count was also monitored on MRS (Himedia, Mumbai, India) agar plates supplemented with 0.1% bromocresol purple. The halophilic bacterial count was enumerated on M73 agar plates (Norberg and Hofsten, 1969). All plates were incubated at 30$^{o}$C for 24-72 h. To monitor the *V. halodenitrificans* MSK-10P, appropriate diluted samples (100 μL) were spread on nutrient agar (NA, Himedia, Mumbai, India) supplemented with 1% skim milk and 10% NaCl. For monitoring *Staph. xylosus* LSM16, appropriate diluted samples (100 μL) were spread on tryptic soy agar (TSA, Himedia, Mumbai, India) supplemented with 1% olive oil (w/v) and 10% NaCl (w/v). Microbiological safety of finished Kapi was investigated by following the online Bacteriological Analytical Manual

(www.fda.gov/food/ucm2006949) and the regulation in Thai Industrial Standard (TIS) 1205/2006 for Kapi product.

### 6.2.4 Enzymatic activities

#### *6.2.4.1 Preparation of aquoeous extract of* Kapi *products*

Kapi samples (10 g) were mixed with 90 mL deionized water. The mixtures were blended at high speed for 1 min, followed by centrifugation at 6800 × *g* for 10 min at 4°C (Sorvall Legend XTR, Thermo Scientific, Langensebold, Germany). The supernatant was collected and adjusted to 100 mL with deionized water. The prepared extracts were assayed for proteolytic and lipolytic activities (Kleekayai *et al*., 2015).

#### *6.2.4.2 Proteolytic activity*

Proteolytic activity was determined by using modified Anson method as previously described in Chapter 2.

#### *6.2.4.3 Lipolytic activity*

Lipase activity was determined titrimetrically using olive oil hydrolysis method (Machedo *et al.*, 1997). One mL of aqueous extract was added to the substrate mixture containing 10 mL of olive oil in 10% iso-octane (w/v), 2 mL of 0.6% (w/v) $CaCl_2$ solution and 5 mL of 50 mM phosphate buffer, pH 7.0. The mixture was incubated on orbital shaker at 200 rpm at 37 ºC for 30 min. Then, 20 mL ethanol-acetone mixture (1:1) was added to stop the reaction. The release fatty acids were titrated against 0.1 N NaOH (phenolphthalein was used as indicator). The activity of lipase was recorded as the amount of the enzyme that can release one μmol oleic acid per min under assay conditions.

### 6.2.5 Determination of biogenic amines

Determination of BAs in final Kapi products and commercial Kapi was performed by using TLC method. At first, samples were prepared for BA extraction according to Kongkiattikajorn (2015) with minor modification. One gram of Kapi

samples was added to 10 mL of 10% TCA and then shaken at 150 rpm for 1 h at room temperature. The samples were further centrifuged at 10,300 × g for 15 minutes. Afterwards, the supernatant was collected for dansyl-derivatization.

The BAs derivatization was performed as described by Landeta *et al*. (2007) and subsequently spotted onto TLC plate (silica gel 60 F254, Merck, Germany). The separation of dansylated compounds were conducted using solvent combination of chloroform:di-ethyl ether:tri-ethylamine (3:2:1, v/v/v). The TLC plate was visualized using a Gel Doc™ EZ (Biorad, London, UK) system under UV light exposure. The authentic BAs stock solutions (histamine, cadaverine, putrescine, and tyrosine) (Sigma-Aldrich, Singapore) were made by preparing a 2% (w/v) solution in 40% ethanol (5% in the case of histamine).

### 6.2.6 Sensory analysis

Sensory evaluation of Kapi was conducted according to Pongsetkul *et al.* (2015). Sensory analysis of Kapi samples was evaluated by 30 panelists who regularly use and consume Kapi. Before served, the samples were heated in oven at 60°C for 30 min. The samples with dimension of approximately 1×1×1 cm was then served in white plate to the panelist. Three digits code was used randomly to mark the samples. Panelists were also directed to wash out their mouths before checking different samples with drinking water and sliced cucumber served with the samples. The panelists were asked for scoring their likeness on samples for appearance, color, aroma, texture, taste and overall acceptance using a 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely) (Mellgard *et al.*, 2007).

### 6.2.7 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using SPSS version 16.0 (SPSS, Illinois, USA). The significant difference of means was determined using the Duncan's Multiple Range Test (DMRT) at a 95% significant difference ($p$<0.05).

## 6.3 Results and Discussions

### 6.3.1 Physicochemical changes during fermentation

Figure 36 shows the physicochemical changes of the Kapi during fermentation. It was found that moisture content drastically decreased from the beginning (T0) until after drying with sunlight (F0) (Figure 36a.). Through salting process, the salt can infiltrate the meat of shrimp which in turn replace the water in the shrimp meat, so that lowered the moisture content (Chaijan, 2011). Combination of the salting and drying process made the moisture content decreased at F0. After first week of fermentation (W1) the moisture content of all four treatments increased and remained stable until the end of fermentation. Sample inoculated with lipase-producing bacteria, *Staph. xylosus* LSM16 shows significantly the highest moisture content (42.73%) at the final stage of fermentation ($p<0.05$), which is coincidence with the paste texture of this sample. Moreover, moisture content of all final products still within the range required and regulated by TIS (≤45%).

Salt content of all Kapi samples had higher at the beginning (T0) and decreased at the F0 (Figure 36b). The high salt content at T0 due to the first addition of sea salt to the mixture and decreased at F0 after sun drying process. Usually after sun-dried the moisture content of samples reduced and the salt content increased however, in this study different result was observed which still unexplainable. After three days sundried (F0), the saline water (5% w/v) usually added by the producer to keep the mixture not very dry which lead the increasing of salt content and remained stable until final fermentation. The salt content of spontaneous Kapi had slightly higher than inoculated Kapi ($p>0.05$).

In this study, the change in pH can be observed for all samples throughout the fermentation process. pH of all treatments increased from the beginning until the end of fermentation (Figure 36c) ($p<0.05$). The increasing of pH throughout the fermentation process was possibly can be correlated with the enzymatic activities of endogenous microorganisms or the starter used, which can form several basic compounds such as ammonia (Pongsetkul *et al.*, 2015). After the fourth week of fermentation, the pH of final products was in the range of 7.34 to 7.43.

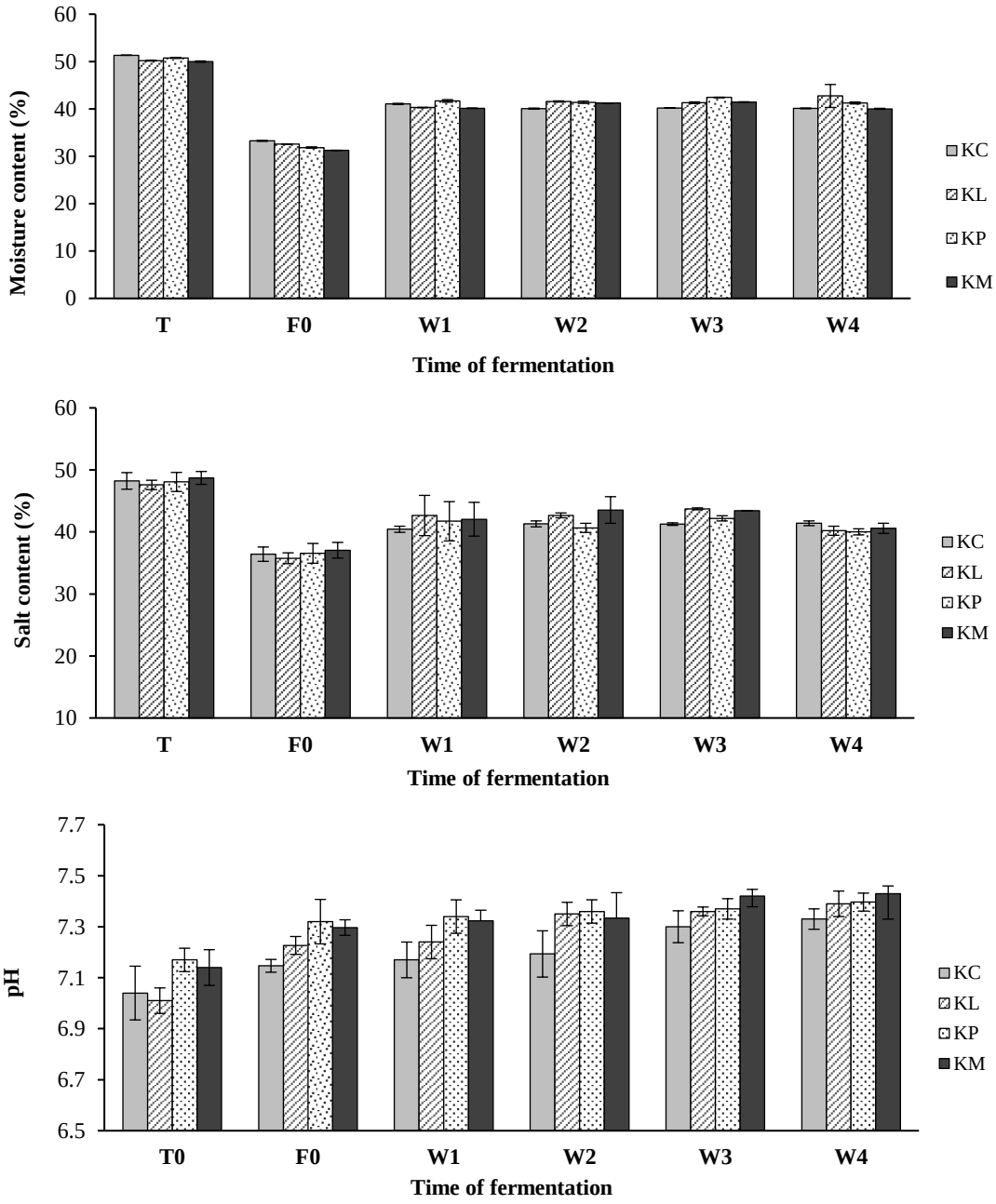



**Figure 36.** Physicochemical change during Kapi fermentation. (a) moisture content; (b) salt content; (c) pH. KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains. T0: day of inoculation of starter cultures; F0: after drying; W1: first week of fermentation; W2: second week of fermentation; W3: third week of fermentation; W4: fourth week of fermentation.

### 6.3.2 Microbiological change of Kapi during fermentation

#### *6.3.2.1 Total viable count and Enterobacteriace*

Figure 37a shows the total bacterial count during fermentation of Kapi. At the beginning of production (T0), total viable count of spontaneous fermented Kapi (KC) was higher than the Kapi inoculated with starter cultures ($p<0.05$). Throughout fermentation, KM had higher TVC count than other treatments. Finally, at the end of fermentation all samples had lower TVC count compared with at the beginning of production process. This is because many microflora can adapt at the beginning environment, while throughout the fermentation the environment was changing; thus, only microflora tolerated to the high salt condition can be survived. In addition, Enterobacteriaceae was not detected throughout the fermentation process. It is because most Enterobacteriaceae was inhibited at the level of 6 – 16 % of NaCl (Wijnker *et al.*, 2006).

Total halophilic count increased from the beginning until the end of fermentation (Figure 37b). Remarkably increasing in number of halophilic bacterial count was observed starting at first week of fermentation and continuously increased until fourth week of fermentation. The high salt environment throughout Kapi processing was plausibly contributed to the proliferation of halophilic bacteria (Kobayashi *et al*., 2003; Chuon *et al*., 2014).

As shown in Figure 37c, LAB count increased on drying process until third week of fermentation. However, the number was constant after 2 weeks ($p<0.05$). The reduction of LAB count at the final stage of fermentation could be influenced by the insufficient substrates of LAB as can be seen by the low amount of carbohydrates (Faithong *et al*., 2010; Klekayai *et al*., 2015; Pongsetkul *et al*., 2014). Halophilic LAB species belonging to *Tetragenococcus*, are found predominantly in various salted-fermented foods (Chuon *et al*., 2014; Kuda *et al*., 2014).

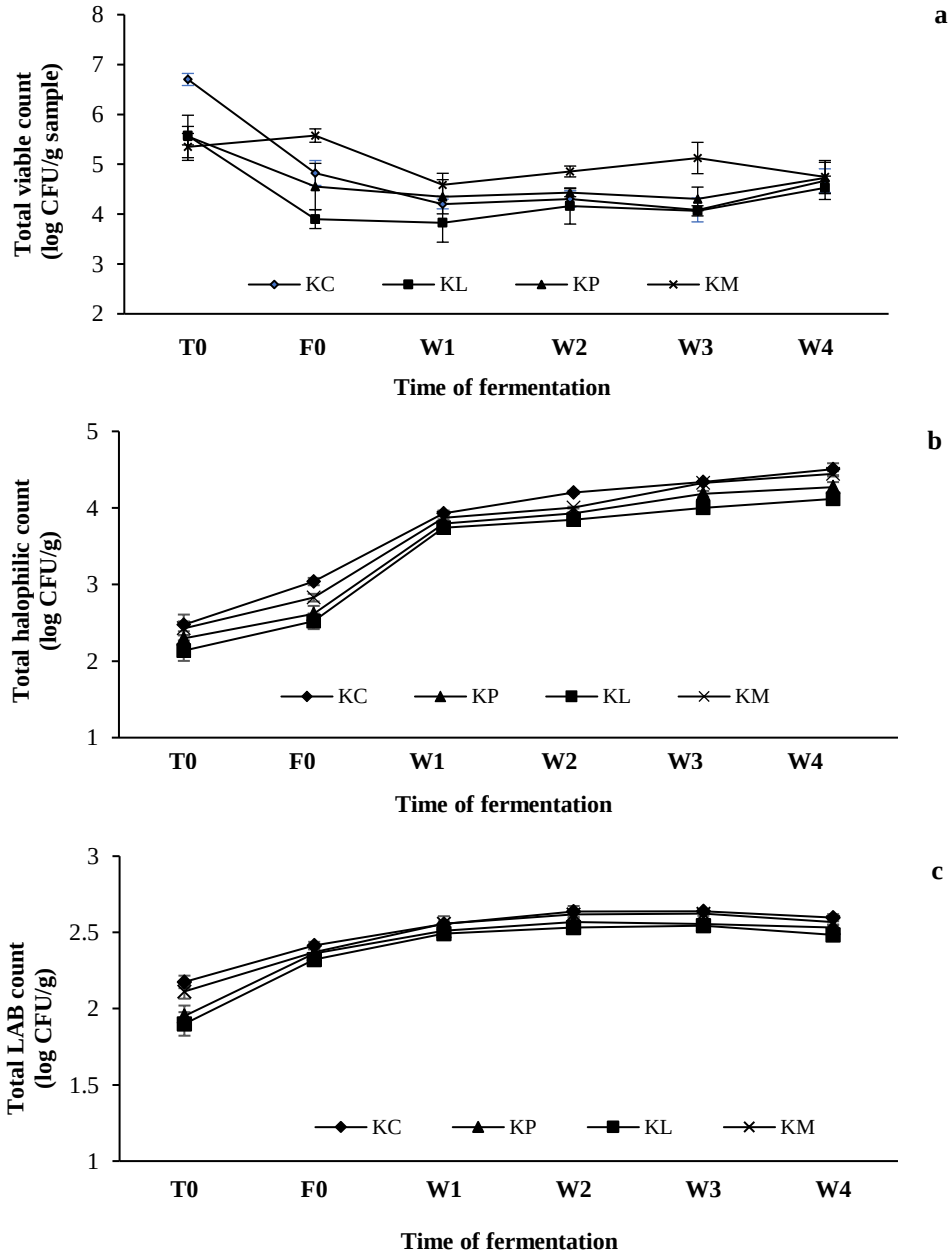



**Figure 37.** Total viable count of Kapi samples during fermentation process (a); total halophilic count (b); LAB count (c). KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains. T0: day of inoculation of starter cultures; F0: after salting and drying; W1: first week of fermentation; W2: second week of fermentation; W3: third week of fermentation; W4: fourth week of fermentation.

#### *6.3.2.2 Proteolytic and lipolytic bacterial count*

Figure 38a shows the proteolytic bacterial changes during Kapi fermentation. Sample inoculated with protease-producing bacteria (KP) had the highest proteolytic bacterial count throughout the fermentation process ($p<0.05$), followed by the sample inoculated with mixed starter inoculation (KM). While sample inoculated with lipase-producing strain (KL) had the lowest proteolytic bacterial count throughout the fermentation process ($p<0.05$). This result indicated that the higher number of proteolytic bacterial count in KP and KM was a result of the proteolytic starter culture being added. However, it can be seen that after W1 and W2 the proteolytic bacterial of KP and KM decrease while KC and KL increase, which presumably because of the increasing of indigenous proteolytic bacteria of the shrimp. In addition, Figure 38b demonstrates the lipolytic bacterial count during the fermentation process. It can be inferred from the figure that sample inoculated with lipase-producing bacteria had the highest lipolytic bacterial count throughout fermentation process ($p<0.05$). Sample inoculated with protease-producing bacteria had the lowest count since the beginning until the end of fermentation. While sample inoculated with mixed starter inoculation and without starter inoculation showed slightly difference in lipolytic bacterial count ($p<0.05$). This was also an indication that the higher number of lipolytic bacterial count in KL was a result of the lipolytic starter culture being added. All the results indicated that the proteolytic and lipolytic starter culture can grow well in the harsh environment during the Kapi fermentation.

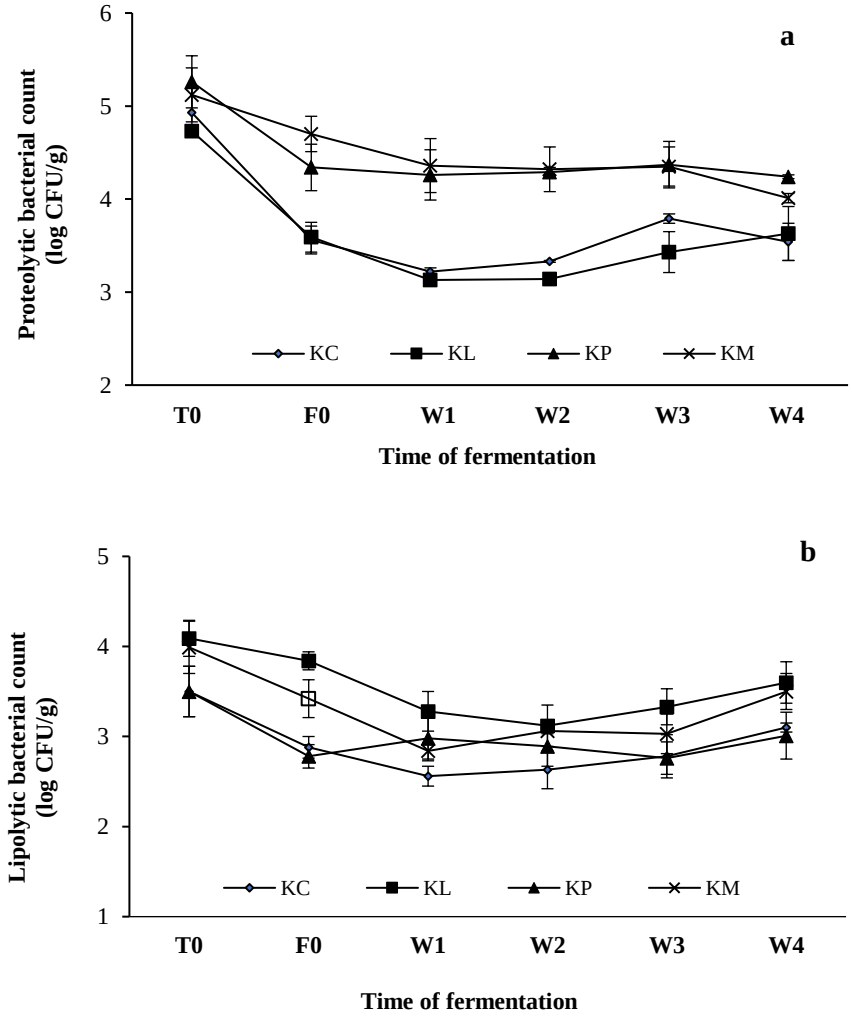



**Figure 38.** Monitoring of: (a) protease-producing bacteria and (b) lipase-producing bacteria during Kapi fermentation. KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains. T0: day of inoculation of starter cultures; F0: after salting and drying; W1: first week of fermentation; W2: second week of fermentation; W3: third week of fermentation; W4: fourth week of fermentation.

### 6.3.3 Comparison of final Kapi product and commercial product

#### *6.3.3.1 Physicochemical characteristic*

The physicochemical properties of Kapi products are shown in Table 19. It can be seen that KL and the commercial Kapi (CP) has the moisture content comparably the same and higher than other products ($p$<0.05). All the moisture content values are still in the value range accepted by TIS 1205-2006. In addition, the pH value of CP was the highest as compared with other samples ($p$<0.05). The pH of all Kapi product was in the range of 7.39-7.43. KC had the highest salt content compared with other treatments and the commercial product had the lowest salt content ($p$<0.05). Although high salt content was detected for all Kapi products in this research, still within the value range accepted by TIS 1205-2006. In contrast, salt content of commercial product did not meet the requirement by TIS 1205-2006 (<36%). High salt was added for product preservation for controlling spoilage and proliferation of pathogenic microorganisms (Pongsetkul *et al.*, 2015). In addition, total nitrogen of all Kapi produced in this research was meet the requirements by TIS 1205-2006 (≥5.8%) however, the commercial product did not meet the requirement (5.36%).

The water activity ($A_w$) of the final Kapi products and commercial product had the range between 0.729 – 0.738. $A_w$ can be associated with the texture of Kapi which vary from soft and pasty to dry and hard. The Kapi product can be categorized as an intermediary moisture food, with $A_w$ about 0.7 (Fennema, 1996). The low $A_w$ of Kapi products could also be related with increase the shelf-life and preserve the product from microbial spoilage at ambient temperature (Goulas and Kontominas, 2005; Prapasuwannakul and Suwannahong, 2015). Moreover, the low $A_w$ could avoid rancidity of the product and limited the growth of food pathogens (Hajeb and Jinap, 2012).

**Table 19.** Characteristics of the fermented Kapi product

| Parameters | | TIS 1205-2006 | Products | | | | |
|---|---|---|---|---|---|---|---|
| | | | KC | KP | KL | KM | CP |
| Moisture (%) | | ≤45 | 40.11±1.40$^{b}$ | 41.25±2.32$^{b}$ | 42.73±2.19$^{a}$ | 40.02±0.93$^{b}$ | 41.60±0.14$^{a}$ |
| pH | | n.a. | 7.34±0.04$^{b}$ | 7.40±0.04$^{b}$ | 7.39±0.05$^{b}$ | 7.43±0.03$^{b}$ | 7.54±0.07$^{a}$ |
| Salt content (%) | | ≥36 | 41.39±0.43$^{a}$ | 40.01±0.43$^{b}$ | 40.19±0.87$^{b}$ | 40.58±0.87$^{ab}$ | 35.72±0.43$^{c}$ |
| Total nitrogen (%) | | ≥5.8 | 7.82±0.78$^{a}$ | 7.10±0.55$^{ab}$ | 6.33±0.50$^{b}$ | 6.98±0.37$^{b}$ | 5.36±0.34$^{c}$ |
| Color | L* | n.a. | 54.32±0.2$^{c}$ | 53.99±0.22$^{c}$ | 53.88±0.12$^{c}$ | 55.50±1.56$^{b}$ | 58.43±0.32$^{a}$ |
| | a* | n.a. | 7.27±0.23$^{c}$ | 7.31±0.22$^{c}$ | 7.22±0.29$^{c}$ | 7.86±0.55$^{b}$ | 9.02±0.21$^{a}$ |
| | b* | n.a. | 8.83±0.15$^{c}$ | 9.44±0.24$^{c}$ | 9.10±0.18$^{c}$ | 10.52±0.99$^{b}$ | 12.32±0.31$^{a}$ |
| | ΔE* | n.a. | 55.51±0.29$^{c}$ | 55.17±0.13$^{c}$ | 55.24±0.26$^{c}$ | 56.76±1.35$^{b}$ | 60.56±0.34$^{a}$ |
| Water activity | | n.a. | 0.729±0.001$^{c}$ | 0.734±0.001$^{bc}$ | 0.736±0.001$^{ab}$ | 0.738±0.002$^{a}$ | 0.736±0.002$^{ab}$ |

Notes: KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains; CP: Commercial product. n.a.: not available. Values are mean ± SD. Different lowercase superscript letters in the same row indicate the significant differences at $p<0.05$.

#### *6.3.3.2 Texture profile analysis*

Table 20 provides the texture analysis of the produced Kapi product compared with the commercial product. Primary characteristics of texture were hardness, adhesiveness, springiness, and cohesiveness, while secondary characteristics were gumminess and chewiness score (Szczesniak *et al.*, 1963; Bourne *et al.*, 1978). It was shown that control sample (KC) without any starter inoculation exhibited high score for the hardness, gumminess and chewiness, which was different with other products ($p<0.05$), while the commercial product had the lowest score in hardness. However, the lowest score for secondary texture parameters, such as gumminess and chewiness were observed in Kapi inoculated with mixed starter (KM). Since KM was inoculated with mixed proteolytic and lipolytic starters, it can be assumed that the activity of protease and lipase produced by the starters take part into lowering the scores of gumminess and chewiness. All the texture profile analysis was conducted to check whether the products could have the same performance as the Kapi available commercially. In addition, all the component analyzed in this texture analysis will affect the acceptance of panelist in sensory analysis test.

**Table 20.** Texture profile analysis of the Kapi product

| Sample | Hardness | Adhesiveness | Springiness | Cohesiveness | Gumminess | Chewiness |
|---|---|---|---|---|---|---|
| KC | 11930.47±407.33$^{a}$ | -209.66±48.87$^{c}$ | 0.31±0.04 $^{c}$ | 0.24±0.02$^{b}$ | 2830.39±247.46$^{a}$ | 874.45±99.58$^{a}$ |
| KP | 9894.32±604.19$^{b}$ | -119.74±32.51$^{b}$ | 0.30±0.02 $^{c}$ | 0.23±0.01$^{b}$ | 2296.51±167.28$^{b}$ | 729.48±38.24$^{b}$ |
| KL | 6342.84±198.83$^{d}$ | -178.16±26.34$^{bc}$ | 0.37±0.08 $^{b}$ | 0.23±0.02$^{b}$ | 1480.59±135.52$^{c}$ | 554.80±79.16$^{c}$ |
| KM | 6557.12±337.94$^{c}$ | -82.12±13.4$^{a}$ | 0.29±0.03$^{c}$ | 0.21±0.01$^{b}$ | 1366.95±81.19$^{d}$ | 391.56±66.2$^{d}$ |
| CP | 6360.44±362.50$^{d}$ | -188.26±76.12$^{c}$ | 0.51±0.18$^{a}$ | 0.27±0.02$^{a}$ | 1432.74±63.89$^{c}$ | 631.38±89.69$^{c}$ |

KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains; CP: Commercial product. Values are mean ± SD. Different lowercase superscript letters in the same row indicate the significant differences at $p<0.05$.

#### *6.3.3.3 Protease and lipase activities*

Table 21 shows the enzymatic activities of final Kapi products compared with commercial product. Kapi inoculated with proteolytic strain had the highest protease activity, followed by commercial Kapi, Kapi inoculated with mixed starter culture, natural fermentation, and Kapi inoculated with lipolytic starter culture, consecutively ($p<0.05$). This result was also supported by the total proteolytic bacterial count (Figure 38a) during fermentation, which was found the highest in KP, followed by KM, KC, and KL, respectively. High proteolytic bacterial count resulted in higher protease activity of the product. Moreover, lipase activity of Kapi inoculated with lipase-producing strain had the highest activity, followed by commercial Kapi, Kapi inoculated with mixed starter culture, natural fermentation, Kapi inoculated with proteolytic starter culture, consecutively ($p<0.05$). This result also related with the lipolytic bacterial count (Figure 38b).

**Table 21.** Protease and lipase activities of final Kapi products and commercial product

| Enzyme activity (U/mL) | Products | | | | |
|---|---|---|---|---|---|
| | KC | KP | KL | KM | CP |
| Protease activity | $18.70\pm0.07^{b}$ | $21.97\pm0.09^{a}$ | $17.60\pm0.27^{c}$ | $18.62\pm0.07^{b}$ | $19.51\pm0.11^{b}$ |
| Lipase activity | $10.04\pm0.35^{c}$ | $9.22\pm1.06^{c}$ | $14.96\pm0.94^{a}$ | $11.47\pm0.35^{b}$ | $11.93\pm0.21^{b}$ |

KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains; CP: Commercial product. Values are mean ± SD. Different lowercase superscript letters in the same row indicate the significant differences at $p<0.05$.

#### *6.3.3.4 Microbiological safety*

Based on data represented in Table 22, it was found that all Kapi products in this study were safe and meet the requirements by TIS 1205-2006. Coliform bacteria were not detected for all produced Kapi and commercial product. In addition, target bacterial pathogens and food-borne pathogens were also not detected in the final

Kapi product and commercial product. Total viable count as well as yeast and mold of Kapi products and commercial product were also under the limitation of TIS 1205-2006. In this study, Kapi was prepared by using high salt content (20% wet basis). High salt environment in Kapi causes lower $A_w$ which then made pathogenic bacteria could not grow, since salt bind to water and reduced the moisture needed by the microorganisms for growing and surviving (Taormina, 2010; Hajeb and Jinap, 2012).

**Table 22.** Microbiological quality of finished Kapi products compared with commercial product

| Parameter | Standard | Products | | | | |
|---|---|---|---|---|---|---|
| | | KC | KP | KL | KM | CP |
| Coliform bacteria (MPN/g) | <3 | <3 | <3 | <3 | <3 | <3 |
| *Staphylococcus aureus* /0.1 g | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Clostridium perfringens* /0.01 g | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Salmonella Typhimurium* /25 g | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Vibrio parahemolyticus* /0.1g | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Total plate count (CFU/g) | $<1 \times 10^5$ | $8.3 \times 10^4$ | $3.8 \times 10^4$ | $5.1 \times 10^4$ | $4.9 \times 10^4$ | $7.4 \times 10^4$ |
| Yeast and Mold (CFU/g) | <50 | 30 | 43 | 30 | 37 | 33 |

n.d.: not detected

KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains; CP: Commercial product.

### 6.3.4 Biogenic amines in samples

The occurrence of BAs in Kapi samples was depicted in Figure 39. It can be inferred from the figure that none of the Kapi samples contained the four important BAs (histamine, tyramine, putrescine, and cadaverine) in seafood samples. This presumably caused by the application of the starter cultures in this study, which were not the BAs producers as concluded from Chapter 2 and 3. Several reports on BAs content in food samples found that the application of non-producer BA could diminished the BAs level in food samples. The addition of *Lactococcus lactis* CPH0-1S as a starter culture in fermented shrimp (kung-som) was markedly reducing accumulation of tyramine (Saelao *et al*., 2018). *Staphylococcus* strains were also having high abilities to inhibit the BAs accumulation in fermented meat (Lu *et al*., 2019). In addition, the co-culture of *Staph. xylosus* and *Lb. plantarum* could inhibit the BAs accumulation in Harbin dry sausages (Sun *et al*., 2016).

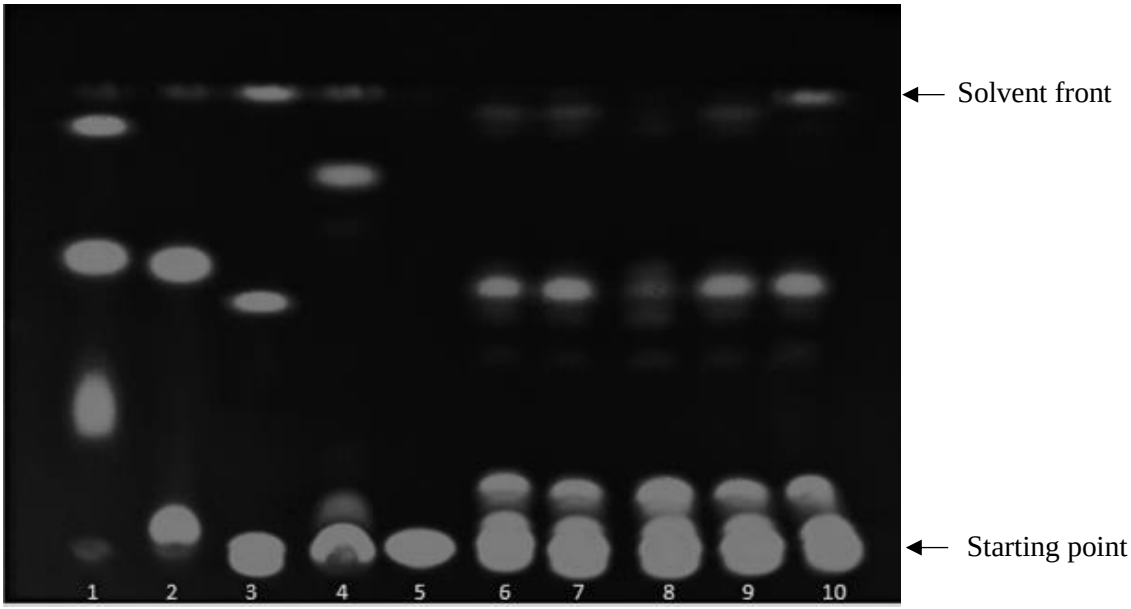



**Figure 39.** Dansylated biogenic amines of standards and samples as separated by TLC. Standard of: tyramine (1), cadaverine (2), putrescine (3), histamine (4); water (5); KC: spontaneous fermentation as a control (6); KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P (7); KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16 (8); KM: inoculated with both strains (9); CP: Commercial product (10).

### 6.3.5 Sensory analysis

Sensory analysis was performed by 30 panelists, with the age range between 25-49 years old. Table 23 summarizes the sensory analysis report of Kapi produced in this research (KC, KP, KL, KM) compared with the commercial product (CP). This table shows that commercial product (CP) had the lowest score in aroma, taste, and overall acceptance by the panelists compared with other Kapi produced in this research ($p<0.05$). However, it should be noted that samples inoculated with single culture and mixed-culture had the highest score for the aroma and taste which means the studied samples get better acceptance compared with the non-starter inoculation and the commercial product. In addition, scores for appearance, color, and texture showed no difference among all samples produced in this study also with the commercial product ($p>0.05$). This reflects that even the inoculated samples had lower value of color (Table 19) and had higher values of texture analysis (Table 20) in some aspects compared with the commercial product, all the Kapi produced in this study can be accepted by the panelists. Moreover, this result provides that the addition of starter culture(s) could produce Kapi better in the sensory quality.

**Table 23.** Sensory analysis of final Kapi products and commercial product

| Sample | Appearance | Color | Aroma | Texture | Taste | Overall |
|---|---|---|---|---|---|---|
| KC | 7.17±0.87[a] | 7.17±0.99[a] | 7.13±1.44[a] | 7.17±1.22[a] | 6.67±1.20[ab] | 6.97±1.31[a] |
| KP | 7.07±1.26[a] | 7.13±1.01[a] | 7.30±1.39[a] | 7.40±1.29[a] | 6.93±1.59[a] | 7.07±0.99[a] |
| KL | 7.30±1.43[a] | 7.20±1.13[a] | 7.23±1.15[a] | 7.23±1.19[a] | 7.03±1.50[a] | 7.17±1.07[a] |
| KM | 7.20±1.45[a] | 7.13±0.90[a] | 7.27±1.08[a] | 7.30±1.25[a] | 6.90±1.47[a] | 7.13±1.20[a] |
| CP | 7.30±1.41[a] | 7.30±1.58[a] | 6.40±1.86[b] | 7.20±1.33[a] | 6.50±1.73[b] | 6.73±1.68[b] |

KC: spontaneous fermentation as a control; KP: inoculated with protease-producing strain, *V. halodenitrificans* MSK-10P; KL: inoculated with lipase-producing strain, *Staph. xylosus* LSM16; KM: inoculated with both strains; CP: commercial product. Values are mean ± SD (n=40). Different lowercase superscript letters in the same column indicate the significant differences at $p<0.05$.

## 6.4 Conclusion

Kapi products inoculated with protease-producing bacteria, *V. halodenirificans* MSK-10P and lipase-producing bacteria, *Staph. xylosus* LSM16, single inoculation and mixed inoculation, met the physicochemical characteristics and microbiological safety required by TIS 1205-2006. Sensory analysis also showed that all Kapi inoculated with those starter culture(s) were well-accepted by panelists compared with Kapi without any starter's inoculation and commercial product. Therefore, protease-producing bacteria, *V. halodenitrificans* MSK-10P and lipase-producing bacteria, *Staph. xylosus* LSM16 could be applied as promising starter cultures in the production of Kapi.

# CHAPTER 7

# CONCLUSIONS AND SUGGESTIONS

## 7.1 Conclusions

1. *V. halodenitrificans* MSK-10P, a selected protease-producing strain isolated from Kapi tolerated to high salt environment as well as exhibited high protease activity. In addition, it was safe to be used as starter culture based on its safety attributes, such as negative activity in hemolysis, neither biofilm formation ability nor biogenic amines production. This strain also exhibited susceptibility to all antibiotics tested.

2. *Staph. xylosus* LSM16, a selected lipase-producing strain showed remarkably functional properties and considerably safe to be applied as starter cultures. It exhibits high lipase activity, considerable salt tolerance, antagonistic activity to several Gram-positive pathogenic bacteria, γ-hemolysis, weak biofilm formation ability, and susceptible to all tested antibiotics. Its antagonistic activity came from bacteriocin-like inhibitory substance. In addition, it was also did not harbor the classical SE genes and genes encoding several important decarboxylase enzymes.

3. Protease production by *V. halodenitrificans* MSK-10P was optimized using OFAT method. Protease production of *V. halodenitrificans* MSK-10P had optimum at temperature 37$^{o}$C, pH 8.0, and agitation at 150 rpm. In addition, 1% (w/v) casein, 2.5% inoculum and 10% NaCl gave the highest protease production. In addition, the highest protease production was obtained at 36 h of incubation with 3.83-fold increased from initial production. Moreover, this protease can be applied in saline environment since it showed stability over 0-25% NaCl.

4. Lipase production by *Staph. xylosus* LSM16 was optimized using OFAT method. Lipase production of *Staph. xylosus* LSM16 showed optimum at temperature 37$^{o}$C, pH 7.0, and agitation at 150 rpm. Moreover, 2% (w/v) olive oil, 1.5% inoculum and 10% NaCl gave the highest lipase production. In addition, 48 h of incubation time exhibited the highest lipase production with 3.34-fold increase of production. Moreover, this protease can also good to be applied in saline environment since it showed stability over 0-20% NaCl.

5. Protease-producing bacteria, *V. halodenitrificans* MSK-10P and lipase-producing bacteria, *Staph. xylosus* LSM16 were studied for their potency as starter cultures in the Kapi production. Application of these strains in single and mixed inoculation produced the Kapi that met the physicochemical characteristics and microbiological safety required by TIS 1205-2006. Sensory analysis also showed that all Kapi inoculated with those starter cultures were well-accepted by panelists compared with spontaneous one and commercial product. In summary, protease-producing bacteria, *V. halodenitrificans* MSK-10P and lipase-producing bacteria, *S. xylosus* LSM16 could be applied as the promising starter cultures in the production of Kapi.

## 7.2 Suggestions

1. Composition of amino acids and volatile compounds of the final Kapi products should also be determined.

2. Purification of protease from *V. halodenitrificans* MSK-10P and lipase from *Staph. xylosus* LSM16 should be performed to further characterize their biochemical properties and investigate their potential application in food biotechnology or other applications in agricultural field.

# REFERENCES

Abol-Fotouh, D.M., Bayoumi, R.A. and Hassan, M.A. 2016. Production of thermoalkaliphilic lipase from *Geobacillus thermoleovorans* DA2 and application in leather industry. Enzyme Res. 7: 1-8.

Adler-Nissen, J. 1993. Proteases. *In* Enzymes in Food Processing. (Tucker, G. and Woods, L., eds.). p. 191–218. Chapman and Hall. New York.

Aiba, S., Humphrey, A.E. and Millis, N.F. 1973. Biochemical Engineering. 2nd ed. Academic Press. New York. p. 92- 127.

Akolkar, A.V., Durai, D. and Desai, A.J. 2010. *Halobacterium* sp. SP1(1) as a starter culture for accelerating fish sauce fermentation. J. Appl. Microbiol. 109: 44-53.

Almeida, L.S., Murata, R.M., Yatsuda, R., Dos Santos, M.H., Nagem, T.J., Alencar, S.M., Koo, H. and Rosalen, P.L. 2008. Antimicrobial activity of *Rheedia brasiliensis* and 7-epiclusianone against *Streptococcus mutans*. Phytomedicine. 15: 886-891.

Alonso, F.O.M., Oliveira, E.B.L., Dellamora-Ortiz, G.M. and Pereira-Meirelles, F.V. 2005. Improvement of lipase production at different stirring speeds and oxygen levels. J. Braz. Sci. Chem. Eng. 22(1): 9–18.

Amoozegar, M.A., Schumann, P., Hajighasemi, M., Fatemi, A.Z. and Karbalaei-Heidari, H.R. 2008. *Salinivibrio proteolyticus* sp. nov., a moderately halophilic and proteolytic species from a hypersaline lake in Iran. Int. J. Syst. Evol. Microbiol. 58: 1159-1163.

Amziane, M., Metiaz, F., Darenfed-Bouanane, A., Djenane, Z., Selama, O., Abderrahmani, A., Cayol, J.L. and Fardeau, M.L. 2013. *Virgibacillus natechei* sp. nov., a moderately halophilic bacterium isolated from sediment of a saline lake in southwest of Algeria. Curr. Microbiol. 66:462–466.

An, S.Y., Asahara, M., Goto, K., Kasai, H. and Yokota, A. 2007. *Virgibacillus halophilus* sp. nov., spore-forming bacteria isolated from soil in Japan. Int. J. Syst. Evol. Microbiol. 57: 1607–1611.

Andrade, V.S., Sarubbo, L.A., Fukushima, K., Miyaji, M., Nishimura, K., Maria, D.E. and Campos-Takaki, G.L. 2002. Production of extracellular proteases by *Mucor circinelloides* using D-glucose as carbon source/substrate. Braz. J. Microbiol. 33: 106-110.

Arora, P.K. 2013. *Staphylococcus lipolyticus* sp. nov., a new cold-adapted lipase producing marine species. Ann. Microbiol. 63: 913- 922.

Asai, K., Yamada, K., Yagi, T., Baba, H., Kawamura, I. and Ohta, M. 2015. Effect of incubation atmosphere on the production and composition of staphylococcal biofilms. J. Infect. Chemother. 21(1): 55-61.

Aslim, B., Yuksekdag, Z. N., Sarikaya, E. and Beyatli, Y. 2005. Determination of the bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish dairy products. LWT-Food Sci. Technol. 38: 691-694.

Association of Official Analytical Chemists (AOAC). 1990. Official Methods of Analysis of the AOAC International. 15th ed. AOAC International. Arlington, Virginia.

Association of Official Analytical Chemists (AOAC). 2005. Official Methods of Analysis of the AOAC International. 18th ed. AOAC International Inc. Gaithersburg, Maryland, USA.

Atsbaha, A.H., Tedla, D.G. and Shfare, M.T. 2017. Phenotypic tests of bacterial antimicrobial susceptibility testing: A Systematic review. SM J. Clin. Med. 3(1): 1020-1026.

Auerswald, L., Morren, C. and Lopata, A.L. 2006. Histamine levels in seventeen species of fresh and processed South African seafood. Food Chem. 98: 231–239.

Babić, I., Markov, K., Kovačević, D., Trontel, A., Slavica, A., Dugum, J., Cvek, D., Svetec, I.K., Posavec, S. and Frece, J. 2011. Identification and characterization of potential autochthonous starter cultures from a Croatian ‘brand’ product ‘Slavonski kulen’. Meat Sci. 88: 517–524.

Bairagi, A., Ghosh, K.S., Sen, S.K. and Ray, A.K. 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacult. Int. 10: 109-121.

Balaban, N. and Rasooly, A., 2000. Staphylococcal enterotoxins. Int. J. Food Microbiol. 61: 1-10.

Battcock, M. and Azam-Ali, S. 1998. Fermented fruits and vegetables: A global perspective. FAO Agricultural Services Bulletin No. 134. FAO United Nations, Rome.

Bayoumi, R.A., El-louboudey, S.S., Sidkey, N.M. and Abd-El-Rahman, M.A. 2007. Production, purification and characterization of thermoalkalophilic lipase for application in bio-detergent industry. J. Appl. Sci. Res. 3: 1752-1765.

Beddows, C.G. 1997. Fermented Fish and Fish Products. *In* Microbiology of Fermented Foods. (Wood, B.J.B., Ed.). p. 416–440. Blackie Academic and Professional. London.

Belicová, A., Mikulášová, M. and Dušinský, R. 2013. Probiotic potential and safety properties of *Lactobacillus plantarum* from Slovak Bryndza cheese. Biomed. Res. Int. 2013: e760298.

Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. 2012. Selection of yeasts as starter cultures for table olives: a step by step procedure. Front. Microbiol. 3: 1–9.

Bertoldo, J.B., Razzera, G., Vernal, J., Brod, F.C., Arisi, A.C. and Terenzi, H. 2011. Structural stability of *Staphylococcus xylosus* lipase is modulated by Zn(2+) ions. Biochim. Biophys. Acta. 1814: 1120–1126.

Bhowmik, S., Islam, S., Ahmed, M.M., Hossain, M.B. and Hossain, M.A. 2015. Protease producing bacteria and activity in gut of tiger shrimp (*Penaeus monodon*). J. Fish. Aquat. Sci. 10: 489-500.

Bidle, K.A., Kirkland, P.A., Nannen, J.L. and Maupin-Furlow, J.A. 2008. Proteomic analysis of *Haloferax volcanii* reveals salinity-mediated regulation of the stress response protein PspA. Microbiol. 154(5): 1436–1443.

Blaiotta, G., Ercolini, D., Pennacchia, C., Fusco, V., Casaburi, A., Pepe, O. and Villani, F. 2004. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products: Evidence for new variants of seG and seI in *S. aureus* AB8802. J. Appl. Microbiol. 97: 719-730.

Blough, B.E., Landavazo, A., Decker, A.M., Partilla, J.S., Baumann, M.H. and Rothman, R.B. 2014. Interaction of psychoactive tryptamines with biogenic amine transporters and serotonin receptor subtypes. Psychopharmacology (Berl.). 231(21): 4135–4144.

Boch, J., Kempf, B., Schmid, R. and Bremer, E. 1996. Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: Characterization of the *gbsAB* genes. J. Bacteriol. 178(17): 5121–5129.

Borresen, E.C., Henderson, A.J., Kumar, A., Weir, T.L. and Ryan, E.P. 2012. Fermented foods: patented approaches and formulations for nutritional supplementation and health promotion. Recent Pat. Food Nutr. Agric. 4: 134-140.

Boskou D. 1996. Olive Oil: Chemistry and Technology. AOCS Press. Champaign, Illinois, USA.

Bouaziz, A., Horchani, H., Ben Salem, N., Gargouri, Y. and Sayari, A. 2011. Expression, purification of a novel alkaline *Staphylococcus xylosus* lipase acting at high temperature. Biochem. Eng. J. 54: 93–102.

Bourne, M.C. 1978. Texture Profile Analysis. Food Technol. 32(7): 62-66.

Bover-Cid, S. and Holzapfel, W.H. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J. Food Microbiol. 53: 33–41.

Bradner, J.R., Gillings, M. and Nevalainen, K.M.H. 1999. Qualitative assessment of hydrolytic activities in Antarctic microfungi grown at different temperatures on solid media. World J. Microbiol. Biotech. 15: 131-132.

Brink, B., Damink, C., Joosten H.M.L.J. and Huis van't Veld, J.H.J. 1990. Occurrence and formation of biologically active amines in foods. Int. J. Food Microbiol. 11(1): 73-84.

Brocchieri, L. 2004. Environmental signatures in proteome properties. Proc. Natl. Acad. Sci. U. S. A. 101(22): 8257–8258.

Brod, F.C., Pelisser, M.R., Bertoldo, J.B., Vernal, J., Bloch, C. Jr., Terenzi, H. and Arisi, A.C. 2010. Heterologous expression and purification of a heat-tolerant *Staphylococcus xylosus* lipase. Mol. Biotechnol. 44: 110–119.

Brooks, J.P. and M.R. McLaughlin. 2009. Antibiotic resistant bacterial profiles of anaerobic swine lagoon effluent. J. Environ. Qual. 38: 2431–2437.

Brown, A.D. 1976. Microbial water stress. Bacteriol. Rev. 40(4): 803.

Cánovas, D., Vargas, C., Kneip, S., Morón, M.J., Ventosa, A., Bremer, E. and Nieto, J.J. 2000. Genes for the synthesis of the osmoprotectant glycine betaine from choline in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. Microbiol. 146(2): 455–463.

Caplice, E. and Fitzgerald, G.F. 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Microbiol. 50: 131–149.

Capozzi, V., Fragasso, M., Romaniello, R., Berbegal, C., Russo, P. and Spano, G. 2017. Spontaneous food fermentations and potential risks for human health. Fermentation. 3: 49-67.

Carelli, D., Centonze, D., Palermo, C., Quinto, M. and Rotunno, T. 2007. An interference free amperometric biosensor for the detection of biogenic amines in food products. Biosens. Bioelectron. 23: 640-647.

Carrasco, I.J., Marquez, M.C. and Ventosa, A. 2009. *Virgibacillus salinus* sp. nov., a moderately halophilic bacterium from sediment of a saline lake. Int. J. Syst. Evol. Microbiol. 59: 3068–3073.

Carson, D.A., Barkema, H.W., Naushad, S. and De Buck, J. 2017. Bacteriocins of non-aureus staphylococci isolated from bovine milk. Appl. Environ. Microbiol. 83: e01015-e01017

Centers for Disease Control and Prevention (CDC). 2013. Antibiotic Resistance Threats in the United States. CDC Threat Report. U.S. Department of Health and Human Services. Atlanta, Georgia, USA.

Chaijan, M. 2011. Physicochemical changes of tilapia (*Oreochromis niloticus*) muscle during salting. Food Chem. 129: 1201–1210.

Chandler, R.E. and McMeekin, T.A. 1989. Combined effect of temperature and salt concentration/water activity on the growth rate of *Halobacterium* spp. J. Appl. Bacteriol. 67: 71-76.

Chang, R.C., Chou, S.J. and Shaw, J.F. 2001. Synthesis of fatty acid esters by recombinant *Staphylococcus epidermidis* lipases in aqueous environment. J. Agric. Food Chem. 49(5): 2619-2622.

Chapin, A., Rule, A., Gibson, K., Buckley, T. and Schwab, K.J. 2005. Airborne multidrug-resistant bacteria isolated from a concentrated swine feeding operation. Environ. Health Perspect. 113(2): 137-42.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. 1998. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J. Food Prot. 61: 1636–1643.

Chauhan, M., Chauhan, R.S. and Garlapati, V.K. 2013. Modelling and optimization studies on a novel lipase production by *Staphylococcus arlettae* through submerged fermentation. Enz. Res. 35: 39–54.

Chen, Q., Kong, B., Han, Q., Liu, Q. and Xu, L. 2016. The role of bacterial fermentation in the hydrolysis and oxidation of sarcoplasmic and myofibrillar proteins in Harbin dry sausages. Meat Sci. 121: 196–206.

Chen, Y.G., Cui, X.L., Fritze, D., Chai, L.H., Schumann, P., Wen, M.L., Wang, Y.X., Xu L.H. and Jiang, C.L. 2008. *Virgibacillus kekensis* sp. nov., a moderately halophilic bacterium isolated from a salt lake in China. Int. J. Syst. Evol. Microbiol. 58: 647–653.

Chen, Y.G., Cui, X.L., Wang, Y.X., Zhang, Y.Q., Tang, S.K., Li, W.J., Liu, Z.X., Wen, M.L. and Peng, Q. 2009a. *Virgibacillus sediminis* sp. nov., a moderately halophilic bacterium isolated from a salt lake in China. Int. J. Syst. Evol. Microbiol. 59: 2058–2063.

Chen, Y.G., Liu, Z.X., Peng, D.J., Zhang, Y.Q., Wang, Y.X., Tang, S.K., Li, W.J., Cui, X.L. and Liu, Y.Q. 2009b. *Virgibacillus litoralis* sp. nov., a moderately halophilic bacterium isolated from saline soil. Antonie van Leeuwenhoek. 96: 323–329.

Chong, C.Y., Abu Bakar, F., Russly, A.R., Jamilah, B. and Mahyudin, N.A. 2011. The effects of food processing on biogenic amines formation. Int. J. Food Res. 18(3): 867–876.

Chowdhury, S.P, Nagarajan, T., Tripathi, R., Mishra, M.N., Le Rudulier, D. and Tripathi, A.K. 2007. Strain specific salt tolerance and osmoregulatory mechanisms in *Azospirillum brasilense*. FEMS Microbiol. Lett. 267: 72–79.

Chu, W.H. 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. J. Ind. Microbiol. Biotechnol. 34(3): 241–245.

Chuon, M.R., Shimoto, M., Koyanagi, T., Sasaki, T., Michihata, T., Chan, S., Mao, S. and Enomoto, T. 2014. Microbial and chemical properties of Cambodian traditional fermented fish products. J. Sci. Food Agric. 94: 1124-1131.

Coenye, T., Peeters, E. and Nelis, H.J. 2007. Biofilm formation by *Propionibacterium acnes* is associated with increased resistance to antimicrobial agents and increased production of putative virulence factors. Res. Microbiol. 158: 386-392.

Collins, J.W., La Ragione, R.M. and Woodward, M.J., Searle, L.E.J. 2009. Application of Prebiotics and Probiotics in Livestock. *In* Prebiotics and Probiotics Science and Technology. (Charalam-popoulos, D. and Rastall, R.A, eds.). p. 1123–1192. Springer Science & Business – Media B.V. New York.

Commission Regulation (EC). 2005. EC 2073/2005 on microbiological criteria for foodstuffs. OJ. (L 338) 1.

Coton, E., Desmonts, M.H., Leroy, S., Coton, M., Jamet, E., Christieans, S., Donnio, P.Y., Lebert, I. and Talon R 2010. Biodiversity of coagulasenegative staphylococci in French cheeses, dry fermented sausages, processing environments and clinical samples. Int. J. Food Microbiol. 137: 221-229.

Cupp-Enyard, C. 2008. Sigma's non-specific protease activity assay – casein as a substrate. J. Vis. Exp. 19: 899.

Da Costa, M.S., Santos, H. and Galinski, E.A. 1998. An overview of the role and diversity of compatible solutes in bacteria and archaea. Adv. Biochem. Eng. Biotechnol. 61: 117–153.

Daroonpunt, R., Tanaka, N., Uchino, M. and Tanasupawat, S. 2018. Characterization and screening of lipolytic bacteria from Thai fermented fish. Sains Malays. 47(1): 91–97.

Daroonpunt, R., Uchino, M., Tsujii, Y., Kazami, M., Oka, D. and Tanasupawat, S. 2016. Chemical and physical properties of Thai traditional shrimp paste (*Kapi*). J. Appl. Pharm. Sci. 6(5): 58-62.

Darvishi, F., Nahvi, I., Zarkesh-Esfahani, H. and Momenbeik, F. 2009. Effect of plant oils upon lipase and citric acid production in *Yarrowia lipolytica* yeast. J. Biomed. Biotechnol. 562943: 1–7.

DasSarma, S. and DasSarma, P. 2012. Halophiles. *In* eLS. John Wiley & Sons, Ltd. Chichester. UK.

Davies, J. and Davies, D. 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74(3): 417–433.

De las Rivas B., Ruiz-Capillas C., Carrascosa A.V., Curiel J.A., Jiménez-Colmenero, F. and Muñoz, R. 2008. Biogenic amine production by Gram-positive bacteria isolated from Spanish dry-cured 'chorizo' sausage treated with high pressure and kept in chilled storage. Meat Sci. 80: 272–277.

den Besten, H.M., Mataragas, M., Moezelaar, R., Abee, T. and Zwietering, M.H. 2006. Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. Appl. Environ. Microbiol. 72: 5884-5894.

Dolliver, H., Gupta, S. and Noll, S. 2008. Antibiotic degradation during manure composting. J. Environ. Qual. 37: 1245–1253.

Drosinos, E. H., Mataragas, M., Xiraphi, N., Moschonas, G., Gaitis, F. and Metaxopoulos, J. 2005. Characterization of the microbial flora from a traditional Greek fermented sausage. Meat Sci. 69: 307–317.

Ebrahimie, E., Ebrahimi, M., Sarvestani, N.R. and Ebrahimi, M. 2011. Protein attributes contribute to halo-stability: Bioinformatics approach. Saline Syst. 7(1): 1-14.

Ebrahimpour, A., Rahman, R. N. Z. R. A., Diana, H. E.C., Basri, M. and Salleh, A.B. 2008. A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic *Geobacillus* sp. strain ARM. BMC Biotechnol. 8(1): 96.

Edupuganti, S., Parcha, L., and Mangamoori, L.N. 2017. Purification and characterization of extracellular lipase from Staphylococcus epidermidis (MTCC 10656). J. Appl. Pharm. Sci. 7(1): 57-63.

EFSA (European Food Safety Authority). 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10: 2740.

EFSA (European Food Safety Authority). 2014. Technical specifications on randomised sampling for harmonised monitoring of antimicrobial resistance in zoonotic and commensal bacteria. EFSA J. 12(5): 3686-3718.

Eichler, J., 2001. Biotechnological uses of archaeal extremozymes. Biotechnol. Adv. 19: 261–278.

Eittenmiller, R.R., Wallis, J.W., Orr, J.H. and Phillips, R.D. 1981. Production of histidine decarboxylase and histamine by *Proteus morganii*. J. Food Prot. 44(11): 815–820.

El Enshasy, H., Abuoul-Enein, A., Helmy, S. and El Azaly, Y. 2008. Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. J. Appl. Sci. 2(3): 583-593.

Ellaiah, P., Srinivasulu, B., Adinarayana, K. 2002. A review on microbial alkaline proteases. J. Sci. Ind. Res. 61: 690-704.

Essid, I. and Hassouna, M. 2013. Effect of inoculation of selected *Staphylococcus xylosus* and *Lactobacillus plantarum* strains on biochemical, microbiological and textural characteristics of a Tunisian dry fermented sausage. Food Control. 32: 707-714.

Essid, I., Ismail, H.B., Hadj Ahmed, S.B., Ghedamsi, R. and Hassouna, M. 2007. Characterization and technological properties of *Staphylococcus xylosus* strains isolated from a Tunisian traditional salted meat. Meat Sci. 77: 204 - 212.

European Commission (EC). 2003. On a generic approach to the safety assessment of microorganisms used in feed/food and feed/food production. A working paper opens for comment. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed_additives_rules_scan-old_report_out178.pdf (26 December 2018).

Evans, M.J. and Cravatt, B.F. 2006. Mechanism-based profiling of enzyme families. Chem. Rev. 106: 3279−3301.

Ezzat, M.A., Zare, D., Karim, R. and Ghazali, H.M. 2015. Trans- and cis-urocanic acid, biogenic amine and amino acid contents in ikan pekasam (fermented fish) produced from Javanese carp (*Puntius gonionotus*) and black tilapia (*Oreochromis mossambicus*). Food Chem. 172: 893–899.

Faithong, N., Benjakul, S., Phatcharat, S. and Binsan, W. 2010. Chemical composition and antioxidative activity of Thai traditional fermented shrimp and krill products. Food Chem. 119:133-140.

Faithong, N.and Benjakul, S. 2012. Changes in antioxidant activities and physico chemical properties of Kapi, a fermented shrimp paste, during fermentation. J. Food Sci. Technol. 51: 2463-2471.

Fan, Y., Tian, L., Xue, Y., Li, Z., Hou, H. and Xue, C. 2017. Characterization of protease and effects of temperature and salinity on the biochemical changes during fermentation of Antarctic krill. J. Sci. Food Agric. 97: 3546-3551.

Feng, D.Q., Zhang, B., Lu, W.D. and Yang, S.S. 2006. Protein expression analysis of *Halobacillus dabanensis* D-8T subjected to salt shock. J. Microbiol. 44(4): 369-374.

Fennema, O.R. 1996. Food Chemistry. 4th ed. Marcell Dekker, Inc. New York. USA.

Flegel, W.A. 2015. Pathogenesis and mechanisms of antibody-mediated hemolysis. Transfusion. 55: S47–S58.

Food and Drug Administration (FDA). 2001 Fish and fisheries products hazards and controls guidance, 3rd ed. US FDA Center for Food Safety and Applied Nutrition, Maryland.

Food and Drug Administration (FDA). 2011. Fish and Fishery Products Hazards and Controls Guidance, 4th ed. Department of Health and Human Services, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington DC.

Fox, L.K., Zadoks, R.N. and Gaskins, C.T. 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infection. Vet. Microbiol. 107: 295-299.

Frank, H.A. and Yoshinaga, D.H. 1984. Histamine Formation in Tuna. *In* Seafood Toxins. Symposium series no. 262. p. 443–451. American Chemical Society, Washington D.C.

Frank, H.A., Yoshinaga, D.H. and Wu, I.P. 1983. Nomograph for estimating histamine formation in skipjack tuna at elevated temperatures. Mar. Fish. Rev. 45: 40–44.

Fukami, K., Funatsu, Y., Kawasaki, K. and Watabe, S. 2004. Improvement of fish sauce odor by treatment with bacteria isolated from the fish-sauce mush (moromi) made from frigate mackerel. J. Food Sci. 69(2): 45-49.

Gad, G.F.M., El-Feky, M.A., El-Rehewy, M.S., Amin, M., Hassan, I. and Abolella, H. 2009. Detection of icaA, icaD genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. J. Infect. Dev. Ctries. 3(5): 342-351.

Ganzle, M.G. and Vogel, R.F. 2003. Studies of the mode of action of reutericyclin, Appl. Environ. Microbiol. 69: 1305–1307.

Gao, P., Jiang, Q., Xu, Y. and Xia, W. 2017. Esterase activities of autochthonous starter cultures to increase volatile flavour compounds in Chinese traditional fermented fish (Suan yu). Int. J. Food Prop. 20: 663–672.

García-Moruno, E., Carrascosa, A.V. and Muñoz R. 2005. A rapid and inexpensive method for the determination of biogenic amines from bacterial cultures by thin-layer chromatography. Food Prot. 68(3): 625-629.

Garcia-Varona, M., Santos, E.M., Jaime, I. and Rovira, J. 2000. Characterization of Micrococcaceae isolated from different varieties of chorizo. Int. J. Food Microbiol. 54: 189-195.

Ghribi, D., Sayari, A., Gargouri, Y. and Bezzine, S. 2009. Improvement of *Staphylococcus xylosus* lipase production through optimization of the culture conditions. Eur. J. Lipid Sci. Technol. 111(10): 967–971.

Goulas, A.E. and Kontominas, M.G. 2005. Effect of salting and smoking method on the keeping quality of chub mackarel (*Scomber japonicus*): biochemical and sensory attributes. Food Chem. 93: 511- 550.

Guimarães, F., Nóbrega, D.B. Richini-Pereira, V.B., Marson, P.M., de Figueiredo Pantoja, J.C. and Langoni, H. 2013. Enterotoxin genes in coagulase-negative and coagulase-positive staphylococci isolated from bovine milk. J. Dairy Sci. 96: 2866–2872.

Glass, K.A., Loeffelholz, J.M., Ford, J.P. and Doyle, M.P. 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented dry sausage. Appl. Environ. Microbiol. 58: 2513-2516.

Gotz, F., Bannerman, T. and Schleifer, K.H. 2006. The Genera *Staphylococcus* and *Macrococcus*. *In* Procaryotes. (Dworkin, M. ed.). Vol. 4. Springer-Verlag. New York. p. 5–75.

Gotz, F., Verheij, H.M. and Rosenstein, R. 1998. Staphylococcal lipases: molecular characterisation, secretion, and processing. Chem. Phys. Lipids. 93(1–2): 15–25.

Guan, L., Cho, K.H. and Lee, J.H. 2011. Analysis of the cultivable bacterial community in jeotgal, a Korean salted and fermented seafood, and identification of its dominant bacteria. Food Microbiol. 28:101–113.

Gupta, A., Joseph, B., Mani, A. and Thomas, G. 2008. Biosynthesis and properties of an extracellular thermostable serine alkaline protease from *Virgibacillus pantothenticus*. World J. Microbiol. Biotechnol. 24: 237-243.

Gupta, R., Beg, Q.K. and Lorenz P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59: 15-32.

Hajeb, P. and Jinap, S. 2012. Fermented shrimp paste products as source of umami in Southeast Asia. J. Nutr. Food Sci. S10: 006.

Halasz A., Barath A., Simon-Sarkadi L. and Holzapfel W.H. 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Technol. 5(2): 42-49.

Hall-Stoodley, L., Stoodley, P., Kathju, S., Hoiby, N., Moser, C., Costerton, W.J, Moter, A. and Bjarnsholt, T. 2012. Towards diagnostic guidelines for biofilm-associated infections. FEMS Immunol. Med. Microbiol. 65: 127–145.

Hammes, W.P. and Knauf, H.J. 1994. Starters in the processing of meat products. Meat Sci. 36(1-2): 155-168.

Hansen, E. 2002. Commercial bacterial starter cultures for fermented foods for the future. Int. J. Food Microbiol. 78: 119-131.

Hechard, Y. and Sahl, H.G. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria Biochimie. 84: 545–557.

Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. and Logan, N.A. 1998. *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus*. Int. J. Syst. Bacteriol. 48: 99–106.

Heyrman, J., Logan, N.A., Busse, H.J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., Swings, J. and De Vos, P. 2003. *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus*. Int. J. Syst. Evol. Microbiol. 53: 501–511.

Holzapfel, W.H. 1997. Use of starter cultures in fermentation on a household scale. Food Control. 8: 241-258.

Holzapfel, W.H. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 75: 197– 212.

Hong, J., Quan, L.H., Heu, S., Jung, K.S., Han, S.W., Moon, E. and Roh, E. 2014. A new antimicrobial substance produced by *Staphylococcus pasteuri* isolated from vegetables. Food Sci. Biotechnol. 23: 983–990.

Horchani, H., Aissa, I., Ouertani, S., Zarai, Z., Gargouri, Y. and Sayari, A. 2012. Staphylococcal lipases: biotechnological applications. J. Mol. Catal. B: Enzym. 76: 125–132.

Horchani, H., Mosbah, H., Ben Salem, N., Gargouri, Y. and Sayari, A. 2009. Biochemical and molecular characterisation of a thermoactive, alkaline and detergent-stable lipase from a newly isolated *Staphylococcus aureus* strain, J. Mol. Catal. B: Enzym. 56: 237–245.

Hu, C., Xiong, N., Zhang, Y., Rayner, S., Chen, S. 2012. Functional characterization of lipase in the pathogenesis of *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. 419: 617–620.

Hua, N.P., Hamza-Chaffai, A., Vreeland, R.H., Isoda, H. and Naganuma, T. 2008. *Virgibacillus salariu*s sp. nov., a halophilic bacterium isolated from a Saharan Salt Lake. Int. J. Syst. Evol. Microbiol. 58: 2409–2414.

Hungerford, J.M. and Arefyev, A.A. 1992. Flow-injection assay of enzyme inhibition in fish using immobilized diamine oxidase. Anal. Chim. Acta. 261: 351-359.

Hwanhlem, N., Buradaleng, S., Wattanachant, S., Benjakul, S., Tani, A. and Maneerat, S. 2011. Isolation and screening of lactic acid bacteria from Thai traditional fermented fish (Plasom) and production of Plasom from selected strains. Food Control. 22: 401-407.

Ibe, A., Saito, K., Nakazato, M., Kikuchi, Y., Fujinuma, K. and Nishima, T. 1991. Quantitative determination of amines in wine by liquid chromatography. J. AOAC Int. 74(4): 695–698.

Ibrahim, D., Weloosamy, H. and Lim, S.H. 2015. Effect of agitation speed on the morphology of *Aspergillus niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation. World J. Biol. Chem. 6(3): 265-271.

Irlinger, F. 2008. Safety assessment of dairy microorganisms: Coagulase-negative staphylococci. Int. J. Food Microbiol. 126: 302-310.

Jaeger, K., Dijkstra, B.W. and Reetz, M.T. 1999. Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases. Annu. Rev. Microbiol. 53: 315-351.

Jain, A. and Agarwal, A. 2009. Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. J. Microbiol. Methods. 76(1): 88– 92.

Jeanson, S., Floury, J., Gagnaire, V., Lortal, S. and Thierry, A. 2015. Bacterial colonies in solid media and foods: a review on their growth and interactions with the micro-environment. Front. Microbiol. 6: 1284-1284.

Jen-Kuo Yanga, Ing-Lung Shihb, Yew-Min Tzengc, San-Lang Wang. 2000. Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. Enz. Microb. Technol. 26: 406–413.

Jeon, C.O., Kim, J.M., Park, D.J., Xu, L.H., Jiang, C.L., Kim, C.J. 2009. *Virgibacillus xinjiangensis* sp. nov., isolated from a Salt Lake of Xin-jiang Province in China. Int. J. Microbiol. 47: 705–709.

Jeong, D.W., Han, S. and Lee, J.H. 2014. Safety and technological characterization of *Staphylococcus equorum* isolates from jeotgal, a Korean high-salt-fermented seafood, for starter development. Int. J. Food Microbiol. 188: 108–115.

Jeong, D.W., Lee, B., Her, J.Y., Lee, K.G. and Lee, J.H. 2016. Safety and technological characterization of coagulase-negative staphylococci isolates from traditional Korean fermented soybean foods for starter development. Int. J. Food Microbiol. 236: 9-16.

Jeya Shakila, R., Vasundhara, T.S. and Kumudavally, K.V. 2001. A comparison of the TLC-densitometry and HPLC method for the determination of biogenic amines in fish and fishery products. Food Chem. 75: 255-259.

Kampfer, P., Arun, A.B., Busse, H.J., Langer, S., Young, C.C., Chen, W.M., Syed, A.A. and Rekha, P.D. 2011. *Virgibacillus soli* sp. nov., isolated from mountain soil. Int. J. Syst. Evol. Microbiol. 61: 275–280.

Khairina, R., Fitrial, Y., Satrio, H. and Rahmi, N. 2016. Physical, chemical, and microbiological properties of “Ronto” a traditional fermented shrimp from South Borneo, Indonesia. Aquat. Procedia. 7: 214-220.

Khoramnia, A., Lai, O.M., Ebrahimpour, A., Tanduba, C.J., Voon, T.S. and Mukhlis, S. 2010. Thermostable lipase from a newly isolated *Staphylococcus xylosus* strain: process optimization and characterization using RSM and ANN. Electr. J. Biotechnol. 13: 5.

Kim, J., Jung, M.J., Roh, S.W., Nam, Y.D., Shin, K.S. and Bae, J.W. 2011. *Virgibacillus alimentarius* sp. nov., isolated from a traditional Korean food. Int. J. Syst. Evol. Microbiol. 61: 2851-2855.

Kim, S.W., Hwang, H.J., Xu, C.P., Choi, J.W. and Yun, J.W. 2003. Effect of aeration and agitation on the production of mycelial biomass and exopolysaccharides in an enthomopathogenic fungus *Paecilomyces sinclarii*. Lett. Appl. Microbiol. 36(5): 321-326.

Klare, I., Konstabel, C., Muller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H. and Witte, W. 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of *Lactobacilli, Pediococci, Lactococci*, and *Bifidobacteria*. Appl. Env. Microbiol. 71: 8982-8986.

Kleekayai T, Saetae D, Wattanachaiyingyong O, Tachibana S, Yasuda M, SuntornsukW. 2015. Characterization and in vitro biological activities of Thai traditional fermented shrimp pastes. J. Food Sci. Technol. 52(3): 1839–1848.

Kloos, W., Schleifer, K-H. and Götz, F. 1992. The genus *Staphylococcus*. *In:* The Prokaryotes. (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H., eds.). p. 1369–1420. Springer-Verlag, New York.

Kloos, W.E., Hardie, J.M. and Whiley, R.A. 2001. International Committee on Systematic Bacteriology. Subcommittee on the taxonomy of staphylococci and streptococci. Minutes of the meetings, 17 September 1996. Int. J. Syst. Evol. Microbiol. 51: 717–718.

Kobayashi, T., Kajiwara, M., Wahyuni, M., Kitakado, T. and Hamada-Sato, N. 2003. Isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: a traditional fermented seafood product in Indonesia. J. Gen. Appl. Microbiol. 49: 279-286.

Kongkiattikajorn, J., 2015. Potential of starter culture to reduce biogenic amines accumulation in som-fug, a Thai traditional fermented fish sausage. J. Ethn. Foods. 4: 186-194.

Krämer, R. 2010. Bacterial stimulus perception and signal transduction: Response to osmotic stress. Chem. Rec. 10(4): 217–229.

Kuda, T., Kawahara, M., Nemoto, M., Takahashi, H. and Kimura, B. 2014. In vitro antioxidant and anti-inflammation properties of lactic acid bacteria isolated from fish intestines and fermented fish from the Sanriku Satoumi region in Japan. Food Res. Int., 64: 248-255.

Kuleasan, H. and Cakmakci, M.L. 2002. Effect of reuterin produced by *Lactobacillus reuteri* on the surface of sausages to inhibit the growth of *Listeria monocytogenes* and *Salmonella* spp. Nahrung/Food, 46: 408–410.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33: 1870-1874.

Kuroda, M., A. Yamashita, H. Hirakawa, M. Kumano, K. Morikawa, M. Higashide, A. Maruyama, Y. Inose, K. Matoba, H. Toh, S. Kuhar, M. Hattori, and T. Ohta. 2005. Whole genome sequence of *Staphylococcus saprophyticus* reveals the pathogenesis of uncomplicated urinary tract infection. Proc. Natl. Acad. Sci. USA. 102: 13272–13277.

Kushner, D.J. 1978. Life in high salt and solute concentrations: halophilic bacteria. *In* Microbial Life in Extreme Environments. (Kushner, D.J., ed). p. 317-368. Academic Press. London.

Ladero, V., Calles-Enríquez, M., Fernández, M. and Alvarez, M.A. 2010. Toxicological effects of dietary biogenic amines. Curr. Nutr. Food Sci. 6: 145–156.

Lai, Q., Yuan, J., Gu, L. and Shao, Z. 2009. *Marispirillum indicum* gen. nov., sp. nov., isolated from a deep-sea environment. Int. J. Syst. Evol. Microbiol. 59(6): 1278–1281.

Lam, M.Q., Nik Mut, N.N., Thevarajoo, S., Chen, S.J., Selvaratnam, C., Hussin, H., Jamaluddin, H. and Chong, C.S. 2018. Characterization of detergent compatible protease from halophilic *Virgibacillus* sp. CD6. 3 Biotech. 8: 104-112.

Lamark, T., Kaasen, I., Eshoo, M.W., Falkenberg, P., McDougall, J. and Strøm, A.R. 1994. DNA sequence and analysis of the bet genes encoding the osmoregulatory choline–glycine betaine pathway of *Escherichia coli*. Mol. Microbiol. 5(5): 1049–1064.

Landeta, G., Curiel, J.A., Carrascosa, A.V., Muñoz, R. and de las Rivas, B. 2013. Characterization of coagulase-negative staphylococci isolated from Spanish dry cured meat products. Meat Sci. 93: 387-396.

Landeta, G., De las Rivas, B., Carrascosa, A.V. and Muñoz, R. 2007. Screening of biogenic amine production by coagulase-negative Staphylococci isolated during industrial Spanish dry-cured ham processed. Meat Sci. 77: 556–561.

Lee, J.S., Lim, J.M., Lee, K.C., Lee, J.C., Park, Y.H. and Kim, C.J. 2006. *Virgibacillus koreensis* sp. nov., a novel bacterium from a salt field, and transfer of *Virgibacillus picturae* to the genus *Oceanobacillus* as *Oceanobacillus picturae* comb. nov. with emended descriptions. Int. J. Syst. Evol. Microbiol. 56: 251–257.

Lee, K.P. and Kim, H.K. 2015. Transesterification reaction using *Staphylococcus haemolyticus* L62 lipase crosslinked on magnetic microparticles. J. Mol. Catal. B: Enzym. 115: 76-82.

Lee, S.J., Lee, Y.J., Jeong, H., Lee, S.J., Lee, H.S., Pan, J.G., Kim, B.C. and Lee, D.W. 2012. Draft genome sequence of *Virgibacillus halodenitrificans* 1806. J. Bacteriol. 194: 6332-6333.

Lee, S.Y. and Rhee, J.S. 1993. Production and partial purification of a lipase from *Pseudomonas putida* 3SK. Enz. Microb. Technol. 15: 617-624.

Lee, S.Y., Kang, C.H., Oh, T.K. and Yoon, J.H. 2012. *Virgibacillus campisalis* sp. nov., from a marine solar saltern. Int. J. Syst. Evol. Microbiol. 62: 347–351.

Lehane, L. and Olley, J. 2000. Histamine fish poisoning revisited. Int. J. Food Microbiol. 58:1–37.

Leroy, F., Verluyten, J. and De Vuyst, L. 2006. Functional meat starter cultures for improved sausage fermentation. Int. J. Food Microbiol. 106: 270 – 285.

Leroy, F., Verluyten, J. and De Vuyst, L. 2006. Functional meat starter cultures for improved sausage fermentation. Int. J. Food Microbiol. 106(3): 270-285.

Lestari, S.D., Herpandi and Simamora, G.R.R. 2017. Effects of different Pediococcus halophilus level and fermentation time on chemical properties of fermented anchovy paste “terasi ikan”. IOP Conf. Series: Materials Science and Engineering. p. 193-199.

Leuko, S., Raftery, M.J., Burns, B.P., Walter, M.R. and Neilan, B.A. 2009. Global protein level responses of *Halobacterium salinarum* NRC-1 to prolonged changes in external sodium chloride concentrations. J. Proteome Res. 8(5): 2218–2225.

Levy, S.B. 1998. “The challenge of antibiotic resistance.” Sci. Am. 278(3): 46-53.

Levy, S.B. and Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges, and responses. Nat. Med. 10: S122-129.

Levy, S.B. and Miller, R. 1989. Gene transfer in the environment. McGraw-Hill Publishing Company. New York.

Li, L., Zou, D., Ruan, L., Wen, Z., Chen, S., Xu, L. and Wei, X. 2019. Evaluation of the biogenic amines and microbial contribution in traditional Chinese sausages. Front Microbiol. 10: 872-882.

Lindgren, S.E. and Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87: 149–164.

López-López, A., Pujalte, M.J., Benlloch, S., Mata-Roig, M., Rosselló-Mora, R., Garay, E. and Rodríguez-Valera, F. 2002. *Thalassospira lucentensis* gen. nov., sp. nov., a new marine member of the alpha-Proteobacteria. Int. J. Syst. Evol. Microbiol. 52(4): 1277–1283.

Lorca, G.L. and de Valdez, G.F. 2009. *Lactobacillus* Stress Responses. *In Lactobacillus* Molecular Biology: From Genomics to Probiotics. (Ljungh, T. and Wadstrom, T. Eds.). p. 115–138. Caister Academic Press. Norfolk. UK.

Madigan, M.T. and Marrs, B.L. 1997. Extremophiles. Sci. Am. 276(4): 82–87.

Maijala R.L., Eerola S.H., Aho M.A. and Hirn J.A., 1993. The effect of GDL-induced pH decreases on the formation of biogenic amines in meat. J. Food Prot. 56(2): 125-129.

Malherbe, S., Bauer, F. and Du Toit, M. 2007. Understanding problem fermentations–A review. S. Afr. J. Enol. Vitic. 28: 169-185.

Marco M. L., Heeney D., Binda S., Cifelli C. J., Cotter P. D., Foligné B., Ganzle, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E.J. and Hutkins, R. 2017. Health benefits of fermented foods: microbiota and beyond. Curr. Opin. Biotechnol. 44: 94–102.

Marklinder, I. and Lönner, C. 1992. Fermentation properties of intestinal strains of *Lactobacillus*, of a sour dough and of a yoghurt starter culture in an oat-based nutritive solution. Food Microbiol. 9: 197–205.

Martín, A., Colin, B., Aranda, E., Benito, M. J. and Cordoba, M.G. 2007. Characterization of *Micrococcaceae* isolated from Iberian dry-cured sausages. Meat Sci. 75: 696–708.

Martineau, F., Picard, F.J., Ke, D., Paradis, S., Roy, P.H., Ouellette, M. and Bergeron, M.G. 2001. Development of a PCR assay for identification of Staphylococci at genus and species levels. J. Clin. Microbiol. 39(7): 2541–2547.

Marty, E., Bodenmann, C., Buchs, J., Hadorn, R., Eugster-Meier, E., Lacroix, C. and Meile, L. 2012. Prevalence of antibiotic resistance in coagulase-negative staphylococci from spontaneously fermented meat products and safety assessment for new starters. Int. J. Food Microbiol. 159: 74-83.

Mazutti, M., Ceni, G., Di Luccio, M. and Treichel, H. 2007. Production of inulinase by solid-state fermentation: effect of process parameters on production and preliminary characterization of enzyme preparations. Bioprocess Biosyst. Eng. 30: 297-304.

Medina, M.A., Urdiales, J.L., Rodríguez-Caso, C., Ramírez, F.J. and Sánchez-Jiménez, F. 2003. Biogenic amines and polyamines: similar biochemistry for different physiological missions and biomedical applications. Crit. Rev. Biochem. Mol. Biol. 38: 23–59.

Mellgard, M.C., Civille G.V., Carr, B.T. 2007. Sensory evalution of Food: Principles and Practices. *In* Sensory Evaluation Techniques. (Mellgard, M.C., ed.). p. 82-88. CRC Press. New York.

Mizutani, T., Kimizuka, A., Ruddle, K. and Ishige, N. 1992. Chemical components of fermented fish products. J. Food Compost. Anal. 5: 152–159.

Mohamed, R., Livia, S., Hassan, S., Soher, E. and Ahmed-Adel, E.B. 2009. Changes in free amino acids and biogenic amines of Egyptian salted-fermented fish (Feseekh) during ripening and storage. Food Chem. 115: 635–638.

Moll, G.N., Konings, W.N. and Driessen, A.J.M. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie van Leeuwenhoek, 76: 185–189.

Montriwong, A., Kaewphuak, S., Rodtong, S., Roytrakul, S. and Yongsawatdigul, J. 2012. Novel fibrinolytic enzymes from *Virgibacillus halodenitrificans* SK1-3-7 isolated from fish sauce fermentation. Process Biochem. 47: 2379-2387

Møretrø, T., Hermansen, L., Holck, A.L., Sidhu, M.S., Rudi, K. and Langsrud, S. 2003. Biofilm formation and the presence of the intercellular adhesion locus *ica* among Staphylococci from food and food processing environments. Appl. Env. Microbiol. 69: 5648-5655.

Moura, T.M., Campos, F.S. and D'Azevedo, P.A. 2012. Prevalence of enterotoxin-encoding genes and antimicrobial resistance in coagulase-negative and coagulase-positive *Staphylococcus* isolates from black pudding in Southern Brazil. Rev. Soc. Bras. Med. Trop. 45(5): 579–585.

Mudgetti, R.E. 1986. Solid-state fermentations. *In*: Microbiology, Manual of Industrial, Domain Biotechnology. (Solmen, A.L., ed.). p. 66–83. American Society for Microbiology. Washington DC.

Naila, A., Flint, S., Fletcher, G., Bremer, P. and Meerdink, G. 2010. Control of biogenic amines in food – Existing and emerging approaches. J. Food Sci. 75: R139-R150.

Namwong, S., Tanasupawat, S., Lee, K.C. and Lee, J.S. 2009. *Oceanobacillus kapialis* sp. nov., from fermented shrimp paste in Thailand. Int. J. Syst. Evol. Microbiol. 59: 2254-2259.

Nath, A., Chaube, R. and Karthikeyan, S. 2012. Discrimination of psychrophilic and mesophilic proteins using random forest algorithm. *In* 2012 International Conference on Biomedical Engineering and Biotechnology (iCBEB). Piscataway, New Jersey. p. 179–182.

Niederberger, T.D., Steven, B., Charvet, S., Barbier, B. and Whyte, L.G. 2009. *Virgibacillus arcticus* sp. nov., a moderately halophilic, endospore-forming bacterium from permafrost in the Canadian high Arctic. Int. J. Syst. Evol. Microbiol. 59: 2219–2225.

Nilegaonkar, S.S., Zambare, W.P., Kanekar, P.P., Dhakephalkar, P.P. and Sarnaik, S.S. 2007. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. Bioresour. Technol. 98: 1238-1245.

Norberg, P. and von Hofsten, B. 1969. Proteolytic enzymes from extremely halophilic bacteria. J. Gen. Microbiol. 55: 251-256.

Nouroozi, A.S., Rezaei, S., Khoshnevis, N., Doosti, M., Hajihoseini, R., Khoshayand, M.R. and Faramarzi, M.A. 2015. Medium based optimization of an organic solvent tolerant extracellular lipase from the isolated halophilic *Alkalibacillus salilacus*. Extremophiles. 19: 933–947.

Oh, Y.J. and Jung, D.S. 2015. Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from Omegisool, a traditionally fermented millet alcoholic beverage in Korea. LWT - Food Sci. Technol. 63: 437-444.

Okuzumi, M., Okuda, S. and Awano, M. 1984. Effects of temperature, pH value and NaCl concentration on histamine formation of N-group bacteria (psychrophilic and halophilic histamine-forming bacteria). Bull. Japan. Soc. Sci. Fish. 50: 1757–1762.

Olesen, P.T., Meyer, A.S. and Stahnke, L.H. 2004. Generation of flavour compounds in fermented sausages-the influence of curing ingredients, *Staphylococcus* starter culture and ripening time. Meat Sci. 66: 675–687.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Verschueren, K.H.G. and Goldman, A. 1992. The Alpha/Beta-Hydrolase Fold. Protein Eng. 5: 197–211.

Omojasola, P.F. and Jilani, O.P. 2009. Cellulase production by *Trichoderma longi*, *Aspergillus niger* and *Saccharomyces cerevisae* cultured on plantain peel. Res. J. Microbiol. 4: 67–74.

Onal, A., Tekkeli, S.E.K. and Onal, C. 2013. A review of the liquid chromatographic methods for the determination biogenic amines in foods. Food Chem. 138: 509–515.

Oren, A. 2008. Microbial life at high salt concentrations: Phylogenetic and metabolic diversity. Saline Syst. 4: 2-15.

Oren, A., Larimer, F., Richardson, P., Lapidus, A. and Csonka, L.N. 2005. How to be moderately halophilic with broad salt tolerance: Clues from the genome of *Chromohalobacter salexigens*. Extremophiles. 9(4): 275–279.

Osman, K.M., Badr, J., Al-Maary, K., Moussa, I., Hessain, A., Girh, Z. and Saad, A. 2016. Prevalence of the antibiotic resistance genes in coagulase-positive-and negative-Staphylococcus in chicken meat retailed to consumers. Front. Microbiol. 7: 1846.

Pakdeeto, A., Tanasupawat, S., Thawai, C., Moonmangmee, S., Kudo, T. and Itoh, T. 2007. *Lentibacillus kapialis* sp. nov., from fermented shrimp paste in Thailand. Int. J Syst. Evol. Microbiol. 57: 364–369.

Pan, B.S. 1985. Histamine formation in canning processes. *In* Histamine Formation in Marine Products: Production by Bacteria, Measurement and Prediction of Formation, FAO Fisheries Technical Paper, No. 252. pp. 41–44. Food and Agriculture Organization of the United Nations, Rome.

Pant, G., Prakash, A., Pavani, J.V.P., Bera, S., Deviram, G.V.N.S., Kumar, A., Panchpuri, M. and Prasuna, R.G. 2015. Production, optimization, and partial purification of protease from *Bacillus subtilis*. J. Taibah Univ. Sci. 9: 50-55.

Papamanoli, E., Kotzekidou, P., Tzanetakis, N. and Litopoulou-Tzanetaki, E., 2002. Characterization of Micrococcaceae isolated from dry fermented sausage. Food Microbiol. 19: 441-449.

Park, M., Walpola, B.C. and Yoon, M.H. 2013. Purification and characterization of protease enzyme from *Burkholderia stabilis*. Afr. J. Biotechnol. 12(12): 1408-1418.

Peng, Q.Z., Chen, J., Zhang, Y.Q., Chen, Q.H., Peng, D.J., Cui, X.L., Li, W.J. and Chen, Y.G. 2009. *Virgibacillus zhanjiangensis* sp. nov., a marine bacterium isolated from sea water. Antonie Van Leeuwenhoek. 96: 645–652.

Peralta, E.M., Hatate, H., Kawabe, D., Kuwahara, R., Wakamatsu, S., Yuki, T. and Murata, H. 2008. Improving antioxidant activity and nutritional components of Philippine salt-fermented shrimp paste through prolonged fermentation. Food Chem. 111: 72–77.

Perin, L.M., Miranda, R.O., Todorov, S.D., de Melo Franco, B.D.G. and Nero, L.A. 2014. Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. Int. J. Food Microbiol. 185: 121–126.

Phithakpol, B. 1993. Fish fermentation technology in Thailand. *In* Fish Fermentation Technology. 4th ed. (Steinkraus, K.H. and Reilly, P.J., eds.). p. 155–166. United Nation University Press. Tokyo.

Phithakpol, B. and Kasetsat, M. 1995. The traditional fermented foods of Thailand. Institute of food research and product development. Kasetsart University. Bangkok.

Piechota, M. Kot, B., Zdunek, E., Mitrus, J., Wicha, J., Katarzyna, W. and Sachanowicz, K. 2014. Distribution of classical enterotoxin genes in staphylococci from milk of cows with- and without mastitis and the cowshed environment. Polish J. Vet. Sci. 17(3): 407-411.

Place, R.B., Hiestand, D., Burri, S. and Teuber, M. 2002. *Staphylococcus succinus* subsp. *casei* subsp. nov., a dominant isolate from a surface ripened cheese. Syst. Appl. Microbiol. 25: 353–359.

Podkowik, M., Seo, K. S., Schubert, J., Tolo, I., Robinson, D. A., Bania, J. and Bystroń, J. 2016. Genotype and enterotoxigenicity of *Staphylococcus epidermidis* isolate from ready to eat meat products. Int. J. Food Microbiol. 229: 52-59.

Pongsetkul, J., Benjakul, S., Sampavapol, P., Osako, K. and Faithong, N. 2014. Chemical composition and physical properties of salted shrimp paste (Kapi) produced in Thailand. Int. Aquat. Res. 6: 155–166.

Pongsetkul, J., Benjakul, S., Sampavapol, P., Osako, K. and Faithong, N. 2015. Chemical compositions, sensory and antioxidative properties of salted shrimp paste (Kapi) in Thailand. Int. Food Res J. 22(4): 1454-1465.

Pongsetkul, J., Benjakul, S., Sumpavapol, P., Vongkamjan, K. and Osako, K. 2018. Quality of Kapi, salted shrimp paste of Thailand, inoculated with *Bacillus* spp. KC-3. J. Aquat. Food Prod. T. 27: 830-843.

Poole, K. 2001. Overcoming antimicrobial resistance by targeting resistance mechanisms. J. Pharm. Pharmacol. 53(3): 283–294.

Prapasuwannakul, N. and Suwannahong, K. 2015. Chemical composition and antioxidant activity of Klongkone shrimp paste. Procedia Soc. Behav. Sci. 197: 1095-1100.

Price, R.J. and Melvin, E.F. 1994. Recommendations for on Board Handling of Albacore Tuna. Sea Grant Extension Program. Food Science and Technology Extension. University of California, Davis.

Price, S. B. and D. J. Flournoy. 1982. Comparison of antimicrobial susceptibility patterns among coagulase-negative staphylococci. Antimicrob. Agents Chemother. 21: 436-440.

Purohit, M.K. and Singh, S.P. 2011. Comparative analysis of enzymatic stability and amino acid sequences of thermostable alkaline proteases from two haloalkaliphilic bacteria isolated from coastal region of Gujarat, India. Int. J. Biol. Macromol. 49(1): 103–112.

Quesada, T., Aguilera, M., Morillo, J.A., Ramos-Cormenzana, A. and Monteoliva-Sanchez, M. 2007. *Virgibacillus olivae* sp. nov., isolated from waste wash-water from processing of Spanish-style green olives. Int. J. Syst. Evol. Microbiol. 57: 906–910.

Raetz C.R. and Whitfield C. 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 71: 635-700.

Rajeswari, V. D., Jayaraman, G. and Sridharan, T. 2012. Purification and characterization of extracellular protease from halotolerant bacterium *Virgibacillus dokdonensis* VITP14. Asian J. Biochem. 7: 123-132.

Rajkumar, H.R.V., Devaki, R. and Kandi, V. 2016. Comparison of hemagglutination and hemolytic activity of various bacterial clinical isolates against different human blood groups. Cureus. 8: e489.

Rall, V.L.M., Sforcin, J.M., Deus, M.F.R., Sousa, D.C., Camargo, V.H., Godinho, N.C., Galindo, L.A., Soares, T.C.S. and Araújo-Junior, J.P. 2010. Polymerase chain reaction detection of enterotoxins genes in coagulase-negative staphylococci isolated from Brazilian minas cheese. Foodborne Pathog. Dis. 7: 1121-1123.

Rantsiou, K., Iacumin, L., Cantoni, C., Comi, G. and Cocolin, L. 2005. Ecology and characterization by molecular methods of *Staphylococcus* species isolated from fresh sausages. Int. J. Food Microbiol. 97: 277-284.

Rao, M.B., Tanksale, A., Ghatge, M. and Deshpande, V. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62(3): 597-635.

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W. and Sukhoom, A. 2013. Selection of γ-aminobutyric acid-producing lactic acid bacteria and their potential as probiotics for use as starter cultures in Thai fermented sausages (Nham). Int. J. Food Sci. Technol. 48: 1371-1382.

Raval, V.H., Bhatt, H.B. and Singh, S.P. 2018. Adaptation strategies in halophilic bacteria. *In* Extremophiles, from Biology to Biotechnology. (Durvasula, R. and Subba Rao, D.V., eds.). p. 137-163. CRC Press. Taylor & Francis Group. Boca Raton, Florida.

Razak, N.A., Samad, M.Y.A., Basri, M., Yunus, W.M.Z.W, Ampon, K. and Salleh, A.B. 1994. Thermostable extracellular protease of *Bacillus stearothermophilus*: factors affecting its production. World J. Microbiol. Biotechnol. 10: 260-263.

Rebecchi, A., Crivori, S., Sarra, P.G. and Cocconcelli, P.S. 1998. Physiological and molecular techniques for the study of bacterial community development in sausage fermentation. J. Appl. Microbiol. 84: 1043–1049.

Reis, P., Holmberg, K., Watzke, H., Leser, M.E. and Miller, R. 2009. Lipases at interfaces: A review. Adv. Colloid Interface Sci. 147-148: 237-250.

Ródriguez-Gómez, F., Romero-Gil, V., Bautista-Gallego, J., Garrido-Fernández, A. and Arroyo-López, F.N. 2012. Multivariate analysis to discriminate yeast strains with technological applications in table olive processing. World J. Microbiol. Biotechnol. 28: 1761–1770.

Rosenstein, R., Futter-Bryniok, D. and Götz, F. 1999. The choline-converting pathway in *Staphylococcus xylosus* C2A: Genetic and physiological characterization. J. Bacteriol. 181(7): 2273–2278.

Sabu, A., Kiran, G.S. and Pandey, A. 2005. Purification and characterization of tannin acyl hydrolase from *Aspergillus niger* ATCC 16620. Food Technol. Biotechnol. 43(2): 133-138.

Saelao, S., Maneerat, S., Thongruck, K., Watthanasakphuban, N., Wiriyagulopas, S. and Chobert, J. M. 2018. Reduction of tyramine accumulation in Thai fermented shrimp (*kung-som*) by nisin Z-producing *Lactococcus lactis* KTH0-1S as starter culture. Food Control. 90: 249–258.

Sangeetha, R., Arulpandi, I. and Geetha, A. 2011. Bacterial lipases as potential industrial biocatalysts: An overview. Res. J. Microbiol., 6:1-24.

Sapkota, A.R., Curriero, F.C., Gibson, K.E. and Schwab, K.J. 2007. Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated swine feeding operation. Environ. Health Perspect. 115(7): 1040–1045.

Sarojnalini, C. and Suchitra, T. 2009. Microbial and nutritional evaluation of fermented *Setipinna* species. Fish Tech. 46(2): 165–170.

Sarrouh, B., Santos, T.M., Miyoshi, A., Dias, R. and Azevedo, V. 2012. Up-to-date insight on industrial enzymes applications and global market. J. Bioprocess. Biotech. S4: 002.

Sashihara, T., Kimura, H., Higuchi, T., Adachi, A., Matsusaki, H., Sonomoto, K. and Ishizaki, A. 2000. A novel lantibiotic, nukacin ISK-1, of Staphylococcus warneri ISK-1: cloning of the structural gene and identification of the structure. Biosci. Biotechnol. Biochem. 64: 2420 –2428.

Sathishkumar, R., Ananthan, G. and Raghunathan, C. 2015. Production and characterization of haloalkaline protease from ascidian associated *Virgibacillus halodenitrificans* RSK CAS1 using marine wastes. Annals Microbiol. 65: 1481-1493.

Sayari, A., Agrebi, N., Jaoua, S. and Gargouri, Y. 2001. Biochemical and molecular characterization of *Staphylococcus simulans* lipases. Biochimie 83: 863–871.

Schleifer, K-H. and W. E. Kloos. 1975. Isolation and characterization of staphylococci from human skin. I: Amended descriptions of *Staphylococcus saprophyticus* and descriptions of three new species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, and *Staphylococcus xylosus*. Int. J. Syst. Bacteriol. 25: 50–61.

Seiler, H. and Wenning, M. 2013. *Virgibacillus halotolerans* sp. nov., isolated from a dairy product. Int. J. Syst. Evol. Microbiol. 63: 3358–3363.

Seitter, M., Geng, B. and Hertel, C. 2011. Binding to extracellular matrix proteins and formation of biogenic amines by food-associated coagulase-negative staphylococci. Int. J. Food Microbiol. 145: 483–487.

Shalaby, A.R. 1996. Significance of biogenic amines to food safety and human health. Food Res. Int. 29: 675–690.

Shi, X. and Zhu, X. 2009. Biofilm formation and food safety in food industry. Trends Food Sci. Technol. 20(9): 407-413.

Silla Santos, M.H. 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. 29: 213–231.

Sim, K.Y., Chye, F.Y. and Anton, A. 2015. Chemical composition and microbial dynamics of budu fermentation, a traditional Malaysian fish sauce. Acta Aliment. 44: 185-194.

Singh, S. and Bajaj, B.K. 2016. Bioprocess optimization for production of thermoalkali-stable protease from *Bacillus subtilis* K-1 under solid state fermentation. Prep. Biochem. Biotechnol. 46: 717–724.

Singh, S. and Bajaj, B.K. 2017. Agroindustrial/forestry residues as substrates for production of thermoactive alkaline protease from *Bacillus licheniformis* K-3 having multifaceted hydrolytic potential. Waste Biomass Valori. 8: 453–462.

Sinha, R. and Khare, S. 2012. Isolation of a halophilic *Virgibacillus* sp. EMB13: Characterization of its protease for detergent application. Indian J. Biotechnol. 11: 416-426.

Sinsuwan, S., Rodtong, S. and Yongsawatdigul, J. 2007. NaCl-activated extracellular proteinase from *Virgibacillus* sp. SK37 isolated from fish sauce fermentation. J. Food Sci. 72: C264–C269.

Soares, J.C., Marques, M.R., Tavaria, F.K., Pereira, J.O., Malcata, F.X. and Pintado, M.M. 2011. Biodiversity and characterization of *Staphylococcus* species isolated from a small manufacturing dairy plant in Portugal. Int. J. Food Microbiol. 146: 123-129.

Søndergaard, A.K. and Stahnke, L.H. 2002. Growth and aroma production by *Staphylococcus xylosus, S. carnosus* and *S. equorum*–a comparative study in model systems. Int. J. Food Microbiol. 75: 99–109.

Sooch, B.S. and Kauldhar, B.S. 2013. Influence of multiple bioprocess parameters on production of lipase from *Pseudomonas* sp. BWS-5. Braz. Arch. Biol. Technol. 56: 716-718.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernandez, M., Lopez, P., de Palencia, P.F., Corbi, A., Trip, H. and Lolkema, J.S. 2010. Biogenic amines in fermented foods. Eur. J. Clin. Nutr. 64: S95-100.

Spergser, J., Wieser, M., Taubel, M., Rosselló-Mora, R.A., Rosengarten, R. and Busse, H.J. 2003. *Staphylococcus nepalensis* sp. nov., isolated from goats of the Himalayan region. Int. J. Syst. Evol. Microbiol. 53: 2007–2011.

Steinkraus, K.H. 2002. Fermentations in world food processing. Compr. Rev. Food Sci. Food Saf. 1: 23-32.

Steinkraus, K.H. 1995. Handbook of indigenous fermented foods (2nd ed). Taylor & Francis. New York.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B. and Svabic-Vlahovic, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Meth. 40: 175–179.

Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirkovic, I. and Ruzicka, F. 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 115: 891-899.

Suganthi, C., Mageswari, A., Karthikeyan, S., Anbalagan, M., Sivakumar, A., & Gothandam, K. M. 2013. Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediment. J. Gen. Eng. Biotechnol. 11: 47-52.

Sun, Q., Chen, Q., Li, F., Zheng, D. and Kong, B. 2016. Biogenic amine inhibition and quality protection of Harbin dry sausages by inoculation with *Staphylococcus xylosus* and *Lactobacillus plantarum*. Food Control. 68: 358-366.

Surono, I. S. and Hosono, A. 1994. Microflora and their enzyme profile in “Terasi” starter. Biosci. Biotechnol. Biochem., 58 (6): 1167–1169.

Suskovic, J., Blazenka, K., Beganovic, J., Pavunc, A.L., Habjanic, K. and Matosic, S. 2010. Antimicrobial activity – the most important property of probiotic and starter lactic acid bacteria. Food Technol. Biotech. 48(3): 296-307.

Szczesniak, A.S. 1963. Classification of textural characteristics. J. Food Sci. 28: 385-389.

Talon, R. and Leroy, S. 2011. Diversity and safety hazards of bacteria involved in meat fermentations. Meat Sci. 89: 303-309.

Talon, R. and Montel, M.C. 1997. Hydrolysis of esters by staphylococci. Int. J. Food Microbiol. 36: 207-214.

Talon, R., Montel, M.C. and Berdague, J.L. 1996. Production of flavour esters by lipases of *Staphylococcus warneri* and *S. xylosus*. Enz. Microb. Technol. 19: 620-622.

Tamang, J.P. and Kailaspathy, K. 2010. Fermented foods and beverages of the world. CRC Press. Florida.

Tamang, J.P., Shin, D.H., Jung, S.J. and Chae, S.W. 2016. Functional properties of microorganisms in fermented foods. Front. Microbiol. 7: 578-590.

Tanasupawat, S., Hashimoto, Y., Ezaki, T., Kozaki, M. and Komagata, K. 1991. Identification of *Staphylococcus carnosus* strains from fermented fish and soy sauce mash. J. Gen. Appl. Microbiol. 37: 479-494.

Tanasupawat, S., Chamroensaksri, N., Kudo, T. and Itoh, T. 2010. Identification of moderately halophilic bacteria from Thai fermented fish (pla-ra) and proposal of *Virgibacillus siamensis* sp. nov. J. Gen. Appl. Microbiol. 56: 369–379.

Tanasupawat, S., Hashimoto, Y., Ezaki, T., Kozaki, M. and Komagata, K. 1992. *Staphylococcus piscifermentans* sp. nov., from fermented fish in Thailand. Int. J. Syst. Bacteriol. 42: 577–581.

Taormina, P.J. 2010. Implications of salt and sodium reduction on microbial food safety. Crit. Rev. Food Sci. Nutr. 50: 209-227.

Tekaia, F., Yeramian, E. and Dujon, B. 2002. Amino acid composition of genomes, lifestyles of organisms, and evolutionary trends: A global picture with correspondence analysis. Gene. 297(1): 51–60.

Temmerman, R., Pot, B., Huys, G. and Swings, J. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81(1): 1-10.

Thai Industrial Standard Institute (TIS). 2006. Thai Community Product Standard (TCPS) 61-2549 for Kapi. Ministry of Industry. Bangkok.

Thayer, D.W., Muller, W.S., Buchanan, R.L. and Phillips, J.G. 1987. Effect of NaCl, pH, temperature, and atmosphere on growth of *Salmonella* Typhimurium in glucose-mineral salts medium. Appl. Environ. Microbiol. 53: 1311-1315.

Til, H.P., Falke, H.E., Prinsen, M.K. and Willems, M.I. 1997. Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. Food Chem. Toxicol. 35(3–4): 337–348.

Tsuzuki, M., Moskvin, O.V., Kuribayashi, M., Sato, K., Retamal, S., Abo, M., Zeilstra-Ryalls, J. and Gomelsky, M. 2011. Salt stress-induced changes in the transcriptome, compatible solutes, and membrane lipids in the facultatively phototrophic bacterium *Rhodobacter sphaeroides*. Appl. Environ. Microbiol. 77(21): 7551–7559.

Tulini, F.L., Winkelstroter, L.K. and De Martinis, E.C.P. 2013. Identification and evaluation of the probiotic potential of *Lactobacillus paraplantarum* FT259, a bacteriocinogenic strain isolated from Brazilian semi-hard artisanal cheese. Anaerobe. 22: 57–63.

Turner, S., Pryer, K.M., Miao, V.P. and Palmer, J.D. 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol. 46: 327-338.

Udo, E.E., Al-Bustan, M.A., Jacob, L.E. and Chugh, T.D., 1999. Enterotoxin production by coagulase-negative staphylococci in restaurant workers from Kuwait City may be a potential cause of food poisoning. J. Med. Microbiol. 48: 819-823.

Udomsil, N., Rodtong, S., Tanasupawat, S. and Yongsawatdigul, J. 2010. Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds. Int. J. Food. Microbiol. 141: 186-194.

Udomsil, N., Rodtong, S., Choi, Y.J., Hua, Y. and Yongsawatdigul, J. 2011. Use of *Tetragenococcus halophilus* as a starter culture for flavor improvement in fish sauce fermentation. *J. Agric. Food Chem.* 59(15): 8401–8408.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. Microbiological Reviews. 56: 395–411.

Vanderbergh, P.A. 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol. Rev. 12: 221–238.

Veciana-Nogués, M.T., Mariné-Font, A. and Vidal-Carou, M.C. 1997. Biogenic amines as hygienic quality indicators of tuna: relationships with microbial counts, ATP-related compounds, volatile amines, and organoleptic changes. J. Agric. Food Chem. 45: 2036-2041.

Vela, J., Hildebrandt, K., Metcalfe, A., Rempel, H., Bittman, S., Topp, E. and Diarra, M. 2012. Characterization of *Staphylococcus xylosus* isolated from broiler chicken barn bioaerosol. Poult. Sci. 91(12): 3003-3012.

Ventosa, A., Nieto, J. J. and Oren, A. 1998. Biology of moderately halophilic aerobic bacteria, Microbiol. Mol. Biol. Rev. 62: 504-544.

Verma, M.L., Azmi, W. and Kanwar, S.S. 2008. Microbial lipases: At the interface of aqueous and non-aqueous media – A review. Acta Microbiol. Immunol. Hung. 55(3): 265–294.

Vieille, C. and Zeikus, G.J. 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. 65(1): 1–43.

Villani, F., Sannino, L., Moschetti, G., Mauriello, G., Pepe, O., Amodio-Cocchieri, R. and Coppola, S. 1997. Partial characterization of an antagonistic substance produced by *Staphylococcus xylosus* 1E and determination of the effectiveness of the producer strain to inhibit *Listeria monocytogenes* in Italian sausages. Food Microbiol. 14: 555–566.

Vohra, A. and Satyanarayana, T. 2002. Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochem. 37: 999–1004.

Wang, C.Y., Chang, C.C., Ng C.C., Chen, T.W. and Shyu, Y.T. 2008. *Virgibacillus chiguensis* sp. nov., a novel halophilic bacterium isolated from Chigu, a previously commercial saltern located in southern Taiwan. Int. J. Syst. Evol. Microbiol. 58: 341–345.

Wang, X., Xue, Y. and Ma, Y. 2010. *Virgibacillus subterraneus* sp. nov., a moderately halophilic Gram-positive bacterium isolated from subsurface saline soil. Int. J. Syst. Evol. Microbiol. 60: 2763-2767.

Wattanakul, U., Sujarit, C., Suwanno, J. and Yupadee, S. 2011. Chemicals and physical quality analysis of shrimp paste (Kapi) in local market. Proceedings of the Annual Academic Conference of Khon Kaen University: Developing the Future of Thai Rural Areas – Stable Foundations for Sustainable Country Development. Khon Khaen, Thailand. 27-29 January 2011. p. 780-783.

Welsh, D.T. 2000. Ecological significance of compatible solute accumulation by micro-organisms: From single cells to global climate. FEMS Microbiol. Rev. 24(3): 263–290.

Whitaker, J.R. 1994. Principle of Enzymology for The Food Science, Second Edition. New York: Marcell-Decker.

Wijnker, J.J., Koop, G. and Lipman, L.J.A. 2006. Antimicrobial properties of salt (NaCl) used for the preservation of natural casings. Food Microbiology 23: 657–662.

Wiley M.S, Sherwood L.M. and Wolverton C.J. 2008. Prescott, Harley and Klein's Microbiology. McGraw Hill, New York.

World Health Organization. 2001. WHO Global Strategy for Containment of Antimicrobial Resistance. Geneva, Switzerland.

World Health Organization. 2014. Antimicrobial Resistance: Global Report on Surveillance 2014. Geneva, Switzerland.

World Health Organization. 2015. Global Action Plan on Antimicrobial Resistance. WHO Document Production Services. Geneva, Switzerland.

Wortel, M.T., Peters, H., Hulshof, J., Teusink, B. and Bruggeman, F.J. 2014. Metabolic states with maximal specific rate carry flux through an elementary flux mode. FEBS J. 281(6): 1547–1555.

Wright, G.D. 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol. 8: 123-128.

Xu, B., Hu, B., Wang, J., Lan, Y., Zhu, Y., Dai, X., Huang, L., Huang, Y. and Du, W. 2018. *Virgibacillus indicus* sp. nov. and *Virgibacillus profundi* sp. nov., two moderately halophilic bacteria isolated from marine sediment by using microfluidic streak plates. Int. J. Syst. Evol. Microbiol. 68: 2015-2023.

Yamanaka, H., Shiomi, K., Kikuchi, T. and Okuzumi, M. 1984. Changes in histamine contents in red meat fish during storage at different temperatures. Bull. Japanese Soc. Sci. Fish. 50: 695–701.

Yankah, V., Ohshima, T. and Koizumi, C. 1993. Effects of processing and storage on some chemical characteristics and lipid composition of a Ghanaian fermented fish product. J. Sci. Food. Agric. 63(2): 227-235.

Yokoi, K.J., Fujii, A., Kondo, M., Kuzuwa, S., Kagaya, S., Yamakawa, A., Taketo, A. and Kodaira, K. 2012. Molecular properties and extracellular processing of the lipase of *Staphylococcus warneri*. J. Mol. Microbiol. Biotechnol. 22: 167–176.

Yongsawatdigul, J., Rodtong, S. and Raksakulthai, N. 2007. Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. J. Food Sci. 72: M382–390.

Yoon, J.H., Kang S.J., Lee, S.Y., Lee, M.H. and Oh, T.K. 2005. *Virgibacillus dokdonensis* sp. nov., isolated from a Korean island, Dokdo, located at the edge of the East Sea in Korea. Int. J. Syst. Evol. Microbiol. 55: 1833–1837.

Yoon, J.H., Kang, S.J., Jung, Y.T., Lee, K.C., Oh, H.W. and Oh, T.K. 2010. *Virgibacillus byunsanensis* sp. nov., isolated from a marine solar saltern. Int. J. Syst. Evol. Microbiol. 60: 291–295.

Yoon, J.H., Oh, T.K. and Park, Y.H. 2004. Transfer of *Bacillus halodenitrificans* Denariaz *et al*. 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov. Int. J. Syst. Evol. Microbiol. 54: 2163–2167.

Yoshida, Y. 1998. Umami taste and tradional seasonings. Food Rev. Int. 14: 213-246.

Zambare, V., Nilegaonkar, S. and Kanekar, P. 2011. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. New Biotechnol. 28(2): 173-181.

Zarei, M., Khezizadeh, M., Kazemipour, S., Hesami, G. and Bemani, E. 2012. Growth and cell morphology of *Listeria monocytogenes* as affected by various concentrations of NaCl and KCl. J. Appl. Biol. Sci. 6: 55-58.

Zhang, G. and Yi, L. 2013. Stability of halophilic proteins: From dipeptide attributes to discrimination classifier. Int. J. Biol. Macromol. 53: 1–6.

Zhang, M., Zhao, C., Lianxiang, D.U., Ping, F. and Chen, L.U. 2008. Expression, purification, and characterization of a thermophilic neutral protease from *Bacillus stearothermophilus* in *Bacillus subtilis.* Sci. China Series C: Life Sci. 51(1): 52-59.

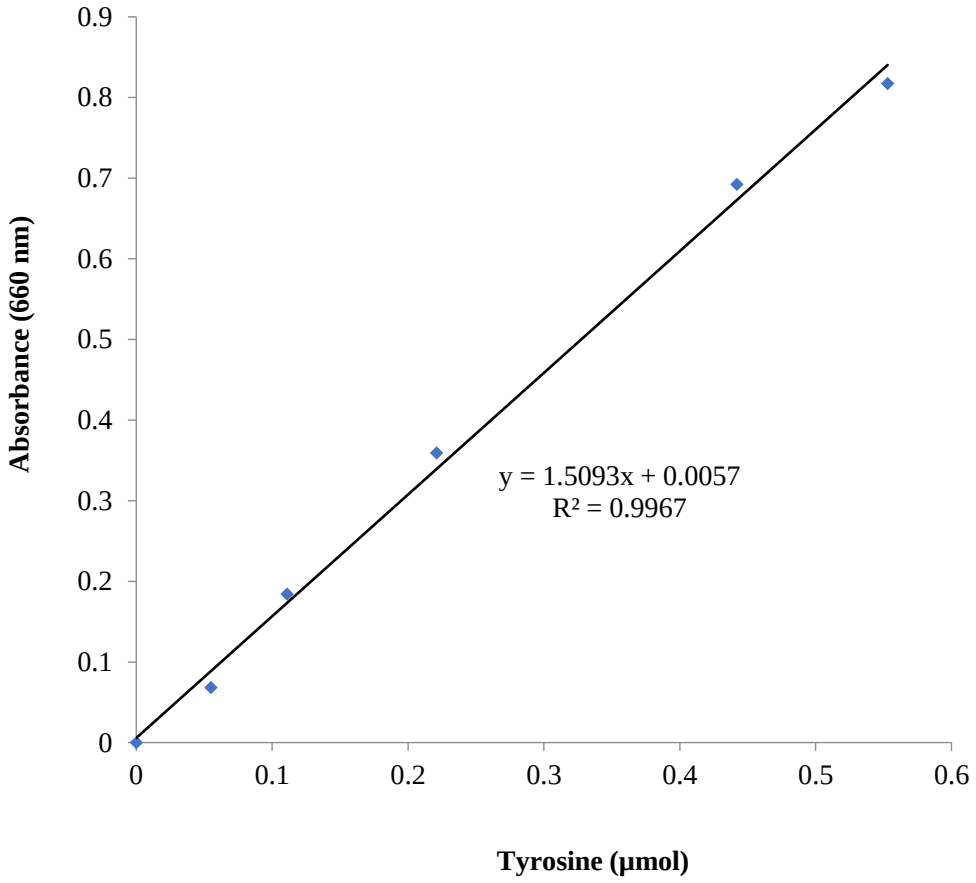
Zhang, Y.J., Zhou, Y., Ja, M., Shi, R., Chun-Yu, W.X., Yang, L.L., Tang, S.K. and Li, W.J. 2012. *Virgibacillus albus* sp. nov., a novel moderately halophilic bacterium isolated from Lop Nur Salt Lake in Xinjiang province, China. Antonie van Leeuwenhoek 102: 553–560.

Zhao, X., Zhao, F., Wang, J. and Zhong, N. 2017. Biofilm formation and control strategies of foodborne pathogens: Food safety perspectives. RSC Adv. 7(58): 36670-36683.

Zhou, Y., Han, L.R., He, H.W., Sang, B., Yu, D.L, Feng, J.T. and Zhang, X. 2018. Effects of agitation, aeration and temperature on production of a novel glycoprotein GP-1 by *Streptomyces kanasenisi* ZX01 and scale-up based on volumetric oxygen transfer coefficient. Molecules. 23: 125.

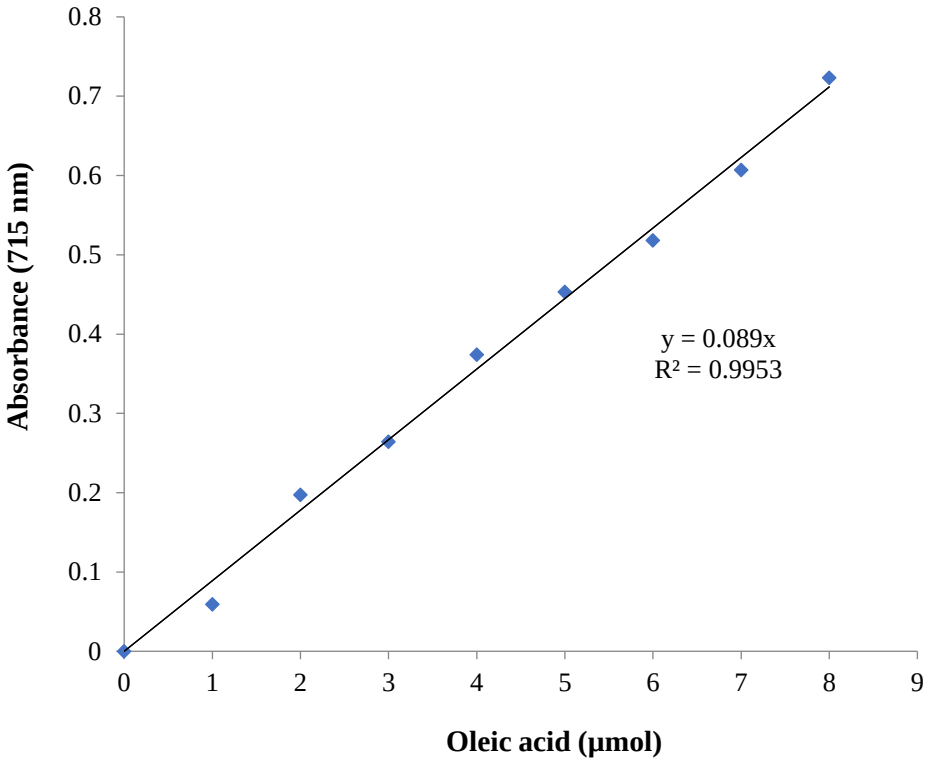
# APPENDIX A

**Standard curve of tyrosine**



# APPENDIX B

## Standard curve for oleic acid



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**List of Publications**

**Kumaunang, M.**, Suyotha, W. and Maneerat, S. 2019. *Virgibacillus halodenitrificans* MSK-10P, a potential protease-producing starter culture for fermented shrimp paste (Kapi) production. (Journal of Aquatic Food Product Technology, 28(8): 877-890. DOI: 10.1080/10498850.2019.1652874).

**Kumaunang, M.**, Suyotha, W. and Maneerat, S. Technological properties and safety attributes of *Staphylococcus xylosus* LSM16, a lipase-producing bacterium as a starter culture candidate in the production of fermented shrimp paste (Kapi). (in preparation).

**Kumaunang, M.**, Suyotha, W. and Maneerat, S. *Virgibacillus halodenitrificans* MSK-10P and *Staphylococcus xylosus* LSM16 as starter cultures in the production of fermented shrimp paste (Kapi). (in preparation).

**Presentations**

**Kumaunang, M.**, Suyotha, W. and Maneerat, S. 2017. Screening, Isolation, and Characterization of Protease- and Lipase-Producing Bacteria Isolated from Fermented Shrimp Paste. The 13th Asian Congress on Biotechnology 2017: "Bioinnovation and Bioeconomy". 23-27 July 2017, Khon Kaen, Thailand (Oral presentation). (http://acb2017thailand.org/userfiles/file_admin/1/Abstract%20book%20of%20ACB%202017_Online%209%20Aug%202017.pdf)