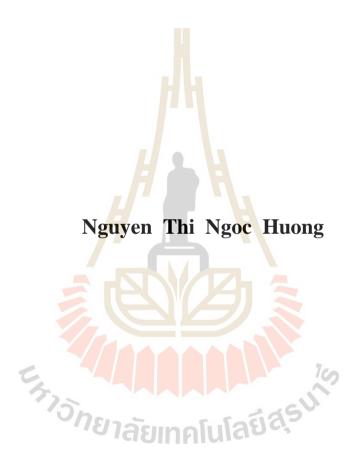
CROSS-PREVENTIVE STRESS RESPONSES ON BACILLUS CEREUS SPORE AND AFLATOXIN B1 IN SAUERKRAUT



A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

Academic Year 2017

การตอบสนองต่อความเครียดร่วมต่อสปอร์ของ *BACILLUS CEREUS* และ AFLATOXIN B1 ในกะหล่ำปลิดอง



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีอาหาร มหาวิทยาลัยเทคโนโลยีสุรนารี ปีการศึกษา 2560

CROSS-PREVENTIVE STRESS RESPONSES ON BACILLUS

CEREUS SPORE AND AFLATOXIN B1 IN SAUERKRAUT

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เหวียน ถิ หงือก เฮื่อง : การตอบสนองต่อความเครียคร่วมต่อสปอร์ของ BACILLUS CEREUS และ AFLATOXIN B1 ในกะหล่าปลีดอง (CROSS-PREVENTIVE STRESS RESPONSES ON BACILLUS CEREUS SPORE AND AFLATOXIN B1 IN SAUERKRAUT) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ปิยะวรรณ กาสลัก, 141 หน้า.

วัตถุประสงค์ของงานวิทยานิพนธ์นี้คือ เพื่อศึกษาอิทธิพลของความเครียคร่วม โดยมีปัจจัย สำคัญ 3 ปัจจัย ได้แก่ เกลือ (1-5% (w/v)) กรคแลคติก (0.5-1.5% (w/v)) และอุณหภูมิระคับกลาง (65-85 องศาเซลเซียส) เป็นเวลา 20 นาที ต่อการตอบสนองความเครียดเชื้อ Bacillus cereus ซึ่งเป็น ้งลินทรีย์ความเสี่ยงสงสคตัวแทนที่สร้างสปอ<mark>ร์ที่</mark>สามารถทนต่อความร้อนและค่าความเป็นกรคค่าง ้ต่ำในผลิตภัณฑ์กะหล่ำปลีดอง ขอบเขตข<mark>องการ</mark>ศึกษาคือ เตรียมสปอร์ของ B. cereus เพื่อทคสอบ กับปัจจัยร่วมดังกล่าวในระดับสภาวะเครียดที่แตกต่างกันภายใต้อาหารเลี้ยงเชื้อเหลว (nutrient broth) เพื่อเขียนกราฟพื้นผิวตอบสนองสามมิติ (Response Surface Methodology) เมื่อผลการ ้ประเมินสภาวะเครียคร่วมทั้ง 3 ปัจจัย <mark>พบ</mark>ว่า สภาว<mark>ะร่ว</mark>มที่เหมาะสมคือปริมาณเกลือ 2% (w/v) กรค แลคติก 1.2% (w/v) และระดับพาสเ<mark>จอ</mark>ไรส์ที่อุณหภูมิ 82 องศาเซลเซียส เป็นเวลา 20 นาที สามารถ ทำลายโครงสร้างเป้าหมายของ<mark>สปอ</mark>ร์ของ B. cereus โ<mark>คยมี</mark>เกณฑ์การพิจารณาร่วม ใค้แก่ การรอค ของสปอร์ การ ใหลของเยื่อหุ้<mark>ม</mark>สปอร์ การเปลี่ยนแปลงสมบัติ<mark>ข</mark>องเยื่อหุ้มสปอร์ที่ยอมให้สารผ่านการ ปลดปล่อยกรดดิพิโคโลน<mark>ิก</mark> การ<mark>ปลดปล่อยดีเอ็นเอ ลักษ</mark>ณะ<mark>ทา</mark>งสันฐานวิทยา และการแสดงออก ของยืนที่ทนต่อสภาวะกรด เกลือ และความร้อน (DnaK hrcA AbrB และ BC1006)

จากนั้นนำสภา<mark>วะเครีย</mark>ดที่เหมาะสมดังกล่าวมา<mark>ทำการ</mark>ทวนสอบเพื่อเป็นขั้นตอนที่ต้อง ควบคุมกระบวนการสำคัญ<mark>ของการหมักกะหล่ำปลีดองและกา</mark>รฆ่าเชื้อด้วยระดับพาสเจอไรส์ โดย เติมเชื้อ B. cereus ในรูปสปอร์ และสารพิษอะฟลาทอกซิน บี 1 ลงไปในสภาวะการหมักแบบ hetero-lactic fermentation ผลการทดลองพบว่าสภาวะเครียดร่วมของเกลือ 2% (w/v) กรดแลกติก 1.2% (w/v) และระดับพาสเจอ ไรส์ที่อุณหภูมิ 82 องศาเซลเซียส เป็นเวลา 20 นาที ยังสามารถควบคุม ปริมาณสปอร์ B. cereus และสามารถลดปริมาณอะฟลาทอกซิน บี 1 ได้ 84.07% (p<0.05) ซึ่ง สอดคล้องกับระดับมาตรฐานความปลอดภัยอาหารด้านจุลินทรีย์ของผลิตภัณฑ์กลุ่มผักดอง

ดังนั้นต้นแบบสภาวะเครียดร่วมดังกล่าวสามารถเป็นวิธีปฏิบัติที่ดีของมาตรการแผนควบคุม และ/หรือ แก้ไขปัญหาการปนเปื้อนปัจจัยเสี่ยงสูงสุดของสปอร์ B. cereus และอะฟลาทอกซิน บี 1 เพื่อกำหนดเป็นวิธีปฏิบัติมาตรฐานด้านสุขาภิบาล (Standard Sanitary Operating Procedure; SSOP) สำหรับอุตสาหกรรมอาหารกลุ่มผลิตภัณฑ์ผักดองที่สนใจต่อไป

สาขาวิชาเทคโนโลยีอาหาร ปีการศึกษา 2560

ลายมือชื่ออาจารย์ที่ปรึกษา_

ลายมือชื่อนักศึกษา

NGUYEN THI NGOC HUONG : CROSS-PREVENTIVE STRESS

RESPONSES ON *BACILLUS CEREUS* SPORE AND AFLATOXIN B1 IN SAUERKRAUT. THESIS ADVISOR :

ASST. PROF. PIYAWAN GASALUCK, Ph.D., 141 PP.

CROSS-PREVENTIVE FACTORS/STRESS ADAPTATION/BACILLUS CEREUS
RESISTANT STRAIN/AFLATOXIN B1/SAUERKRAUT

The purpose of this dissertation was to study the effect of multiple stress factors, such as NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), and mild temperatures (65-85°C) for 20 min on *Bacillus cereus* response, the highest risks are spore forming surrogate and tolerant under heat, and low pH in sauerkraut product. The scope of this study was to prepare the *B. cereus* spores for those combination factors assessed under nutrient broth to demonstrate the Response Surface Methodology (RSM). The accessible results of the three cross-preventive factors used with the appropriate combination factors of 2% (w/v) NaCl, 1.2% (w/v) lactic acid and pasteurization at 82°C for 20 min could damage the *B. cereus* spore target structure, based on combination criteria, which are spore survival, spore membrane fluidity, spore membrane permeability, diplicolonic acid release, DNA release, morphological characteristic, and the expression of acid, salt and heat resistant genes (*Dna*K, *hrc*A, *Abr*B and BC1006).

Afterwards, the appropriate suitable condition was verified as a process critical control measurement of sauerkraut fermentation and its pasteurization, the *B. cereus* spores and aflatoxin B1 were challenged to the hetero-lactic fermentation. The results

revealed that the hurdle combination of 2% (w/v) NaCl and 1.2% (w/v) lactic acid and pasteurization at 82°C for 20 min could still control the level of *B. cereus* spore outgrowth and decreased 84.07% of aflatoxin B1 (p<0.05), which were relative to microbiological safety standard of fermented vegetable products.

Thus, the prototype of the hurdle stress condition could be a good practice as a plan of preventive and/or corrective action to solve the cross-contamination problem of high risk *B. cereus* spore and aflatoxin B1 to specify the Standard Sanitary Operating Procedure (SSOP) for further interest of the fermented vegetable industry.



School of Food Technology

Academic Year 2017

Student's Signature

Advisor's Signature

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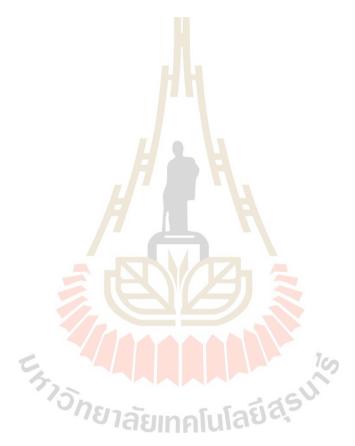
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LIST OF ABBREVIATIONS

 α = Alpha

ANOVA = Analysis of variance

bp = Base pair

 β = Beta

CTAB = Cetyl trimethylammonium bromide

CFU = Colony forming unit

°C = Degree celsius

DNA = Deoxyribonucleic acid

DPA = Dipicolinic acid

et al. et alia (and others)

γ = Gamma

h = Hour

kHz = Kilohertz

(m, μ, p) g, L, mol, M = (milli, micro, pico) Gram, Liter, Mole, Molar

μm = Micrometre

min = Minute

sec = Second

nm = Nanometre

% = Percentage

cm⁻¹ = Reciprocal centimeter

LIST OF ABBREVIATIONS (Continued)

 \times g = Relative centrifugal force

rpm = Revolutions per minute

% (w/w) = Percent mass/mass

% (w/v) = Percent mass/volume

% (v/v) = Percent volume/volume

SSOP = Sanitation Standard Operating Procedure

GMP = Good Manufacturing Practice

HACCP = Hazard Analysis and Critical Control Points

FSMS = Food Safety Management System

CHAPTER I

INTRODUCTION

1.1 Introduction

Nowadays, the requirement of consumers on food safety and food quality has contributed to global emergence of Food Safety Management Systems (Kotsanopoulos and Ioannis, 2017). In Vietnam, food safety is a big matter concern and higher than education, health care or governance (World Bank, 2017). Moreover, food safety supported national economies which contributes to nutrition due to consumer preferences such as traditional food products, vegetable fermentation (pickles) which were produced by lactic acid bacteria (LAB) in brine, namely fermented cucumber, olive and cabbage as a bio-preservation technique has distribute worldwide due to improve food safety and quality of food nutrition. They have unique flavor profiles, crispy texture and are regularly consumed by all age groups, due to its refreshing taste and its status as a healthy food (Hong et al., 2014; Nguyen et al., 2013; Xiong, Guan, Song, Hao, and Xie, 2012; Xia et al., 2017). Inaddition, the lactic and other organic acids (acetic, butyric and propionic acids) are produced by LAB during vegetable fermentation, as well as other metabolic end-products (antioxidant, bacteriocins, sugar, etc) have assured the microbiological safety.

Fermentation of sauerkraut can be divided into hetero-lactic fermentation and homo-lactic fermentation phase, and species and quantity of LAB varies with fermentation stage. The initial phase is hetero-lactic fermentation and dominated by

Leuconostoc citreum, Leuconostoc mesenteroides and Weissella and then gradually transited to homo-lactic fermentation phase, dominated by Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus curvatus, and Lactobacillus. Lactic acid bacteria usually involves the production of antimicrobial compounds with a relatively broad inhibition spectrum (i.e., organic acids, hydrogen peroxide, and nisin). As the recommendation of Food and Drug Administration (FDA) and Codex Alimentarius Austriacus, sauerkraut products have the range of pH (1.5-4.6) and sodium chloride (1-3%) (USDA, 1967; UDSA, 1963; Codex, 2006). Meanwhile, a health benefit is recognized by lowering salt as the recommendation from FDA in commercially processed, packaged, and prepared foods (FDA, 2016), thus bio-preservation of brine vegetable fermentation might be raised by standard quality with intrinsic food safety problems. Moreover, the risk contamination hazards appear at all stages of food chain, fresh fruits and vegetables can be cross-contaminated directly in the field through contact with foodborne pathogens with irrigation water, organic fertilizer by human handling, and during post-harvest transport (Berger et al., 2010; Lynch et al., 2009). In addition, raw cabbages used for fermentation present high microbial loads and may shorten the shelf-life. Amoah et al. (2006) showed the total and fecal coliform contamination through the consumption of fresh vegetables, cabbage were carried ranging from 2.6×10^5 to 1.5×10^{11} CFU/g and 1.4×10^4 to 2.8×10^7 CFU/g wet weight, respectively, whereas cabbage can be contaminated by Bacillus vegetative cells and spores resulting in processing line or post processing contamination (Erickson, 2010; Harris, 2003) which spores are high resistant to various stress than vegetative cells due to their metabolic dormancy and tough physical nature (Jenson and Moir, 2003). Particularly, the survival of high risk hazard of thermophilic spore froming bacteria, which its capacity introduced the multiple stress response in brine condition composed of salt, lactic acid and other acid metabolic activity of during sauerkraut fermentation. The spores are triggered by a process of starvation, and they are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals of which gained the possession of the specific characteristics. The structures of endospores consist of core, cortex, and coat surrounding is surrounded by inner membrane and encompassed by the outer membrane, loosely enclosed by a protein layer called exosporium, respectively. These structures accumulate minerals and dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA) (Atrih and Foster, 2002). DNA is also protected through small acid-soluble proteins. Furthermore, its spores have been shown to be able to adapt to stress (temperature, carbon source, redox potential, pH and osmotic salt (Carlin et al., 2010) by several chromosomal gene of the *B. cereus* contains genes responsible for heat and acid resistances, such as *DnaK*, *hrcA*, *AbrB* (Periago, 2002; Leyn et al., 2013). *B. cereus* spore forming is a one of high risk significant microorganism in sauerkraut that must be, therefore controlled and prevented.

Simultaneously, the contamination in sauerkraut process can be occur because fungi, such as *Aspergillus fumigatus*, *Paecilomyces variotii*, and *Aspergillus flavus*, *Aspergillus niger*. It could cause spoilage of food products after pasteurization, as they are heat resistant. Most molds and yeasts are heat-sensitive and destroyed by heat treatments at temperatures of 140-160°F (60-71°C). Some molds make heat-resistant spores, however, can survive heat treatments in pickled vegetable products. When jars or containers of thermally processed acidified vegetables are improperly sealed or cracked, oxygen may be penetrated. As mentioned before, the heat treated spore survivors may germinate and grow over the liquid surface inside at where the air is

present. There are relatively few spore forming microorganisms which can grow without oxygen and at pH values below 4.6. In addition, *Aspergillus flavus* can produce aflatoxin B1 (Gumus et al., 2010; Jain, 2010) which the most significant contaminant in food and amongst the potent genotoxic and carcinogenic toxins. In case of its toxic nature, Food and Drug Administration (FDA) has set the limiting value for its presence at 20 μg/kg for all foods (USFDA, 2009). In this regard, risk of contamination assessments are an important tool in dealing with food safety issues in order to prevent mycotoxin contamination during fermented food production to gain the good sanitary and hygiene conditions (Adekoya et al., 2017; Inetianbor, 2017).

Recently, most of the sauerkraut product produced in Europe was pasteurized when it reaches a pH of 3.8-4.1 to reduce the microbial contamination for achieving the excessive acidification (Holzapfel et al., 2003). Basicly, bacteria and fungi spore are robust survival vehicles that are highly resistant toward stress conditions, including heat and other stresses during food production and preservation. However, heat treatment is conventionally applied in food processing for microbiological safety as well as, in compliance with consumer demand. Nevertheless, the current food industry trend to use mild temperature treatment together with the reduction of food preservative agents which are sublatally damaged rather than inactivated conceivably causing quality and safety issue after repair and spore outgrowth. The pasteurization is then subjected to apply the sufficient temperature and time to minimize pathogenic and the most spoilage microorganisms (Dubos, 1998). To ensure the safety of acid fermented vegetable, 21 CFR part 120 was established in 2001, whichever regulation mandates a hazards analysis critical control point (HACCP) system with a processing step has been designed to deliver the equivalent to 5-log reduction in sauerkraut. Furthermore, in

order a consideration of suitable processing factors or the environmental safety, shelf life, and quality of food under thermal inactivation parameters are essential for controlling pasteurization process step. At the prior sauerkraut process starting with the function of lactic acid bacteria through facultative fermentation under the presence of NaCl (brine) at 25-30°C until the pH arround 4 to 6 with the procress of sauerkraut characteristics, the finish product would be pasteurized under mild temperature. Through all steps should be performed according to good manufacturing practice (GMP) for ensuring safety product. Meanwile, to assess the high risk significant hazards, especially microbiological and chemical hazards to control and create the corrective action of safety problems in sauerkraut production, it can be designed to prevent the occurrence problems at any point through food safety system.

Generally, the traditional thermal process caused the loss of nutritional value and sensory properties but become excessive acidification, brown and rotten in case of sauerkraut since pasteurization resulting in the vegetative cell inactivation, whereas spore forming bacterial and molds are highly heat resistant and usually unaffected by physical, chemical and biological factors. In order to reduce the undesirable amount of over processing, as well as lethal spore, the development of hurdle technologies to ensure food safety and prolong shelf life of sauerkraut are regared because of multiple hurdle effect sub-lethal shock of cellular component (cell/spore surface, inner membrane, enzyme, protein, RNA, DNA). To ensure sauerkraut product safety, fermented sauerkraut are, therefore, used as the combination key factors of intrinsic (brine, 1-5% (w/v) NaCl, low pH (<4.6) and extrinsic ambient temperatures (25-30°C) or pasteurization (65-85°C) to ensure the microbial safety. The use of combination of sublethal stresses, although synergistically inactivate microorganisms in foods leds to

stress adaptation and cross-protective responses (Juneja and Novak, 2003). Bacteria would be adapted to survive under its stress conditions (Abee and Wouters, 1999). An initial nonlethal heat dose can induce transient resistance to subsequent heat treatment, a phenomenon termed thermotolerance. B. cereus can develop cross-protection from heat after exposure to salt, a low pH, and ethanol, stresses in which common regulators may be involved. Periago et al. (2002) reported the increase in the thermotolerance of B. cereus ATCC 14579 cells exposed to stresses other than heat is not solely protein synthesis dependent. In order that the increased use of mild heat preservation treatments and hurdle technology makes food products more susceptible to bacterial contamination than heavily processed foods. For acid condition, B. cereus displays acid tolerance response when vegetative cells are previously exposed to lower pH, e.g. pH 6.3, they become resistant to normally lethal pH values, for instance pH 4.6 (Jobin et al., 2002; Thomassin et al., 2006). Additionally, exposure to acidic environments may result in enhanced protection when cells are subsequently exposed to lethal heat or hydrogen peroxide stress and a process often referred to as cross-protection (den Besten et al., 2010). Acid tolerance responses and cross-protection phenomena can great implications for controlling B. cereus growth and occurrence in food products and food-processing equipment (Mols and Abee, 2011). Within the concept of hurdle control, it is important to understand that microorganisms can resistant to adverse conditions after a previous stress exposure and thus survive normally lethal conditions that occur during food processing. Furthermore, predictive modelling is an important tool for quantitative determination of the growth and inactivation of microorganisms under specific factors conditions. Recently, only a few predictive microbiological models related to inactivation kinetics of B. cereus and under the combined effects of cross-preventive condition of NaCl and pH and temperatures.

At present, quantitative of corrective and /or preventive action, according to quality assurance systems such as FSMA, QMRA, ISO 31000 and HACCP is a significant approach of which relevant to present microbial hazard in safety risk management. In order to minimize the undesirable amount of over cooking and lethal *B*. cereus spore, the hurdle technology is still remained.

To gain the cross-preventive stress factors potentially imply to SSOP model in sauerkraut production line, multiple hurdle of sub-lethal stress conditions, mild temperatures, acid, and NaCl in controlling B. cereus spore, which were evaluated under the stress spore target changes, such as spore germination rate, membrane fluidity, membrane permeability, DNA release for achieving the sub-lethal condition to control the outgrowth of B. cereus microbiological hazard throughout its risk factors. The predictive microbiological model for the B. cereus under those of cross-preventive condition was applied by using the Response Surface Methodology models. In addition, the safety, and the effectiveness of cross-preventive factor to reduce AFB1 was objectives model. evaluated under laboratory and sauerkraut model.

1.2 Research objectives

To develop, verify, and validate a predictive model for spore germination rate, spore permeability, spore membrane fluidity, DPA release and DNA release of B. cereus spore under cross-preventive factors (NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), temperatures 65-85°C for 20 min).

To evaluate the effect of control measure condition of which achieving the outgrowth of *B.cereus* spore and reducing level of AFB1 under sauerkraut pasteurization.

1.3 Research hypothesis

The cross-preventive factors of NaCl (1, 3, 5% (w/v), lactic acid (0.5, 1.0, 1.5 (w/v)), temperatures (65, 75, 85°C) for 20 min should maximize inhibition level of biological hazards *B.cereus* spore and the reduction of chemical hazards aflatoxin B1. In addition, predictive microbiological modelling might be used for ensuring effectivel control of significant biological and chemical hazards.

1.4 Scope and limitation

The first phase was focused at predictive microbiological modelling of sub-lethal *B. cereus* spore under cross-preventive factors NaCl (1, 3, 5% (w/v), lactic acid (0.5, 1.0, 1.5% (w/v), temperatures (65, 75, 85°C) for 20 min by spore germination rate, spore membrane permeability, spore membrane fluidity, DPA release and DNA release parameters. Afterwards, the mechanism of spore damage under control measure condition was performed by observing the morphology of *B. cereus* spores using Fill emission scanning electron microscope (FE-SEM). Furthermore, conformational changes of functional group of spores were confirmed through Fourier transform infrared spectroscopy (FTIR) analysis. Meanwhile, the heat resistant genes (*Dna*K, *hrc*A, *Abr*B) and acid resistant gene (*Dna*K, BC1006) were determined by polymerase chain reaction (PCR) method based on the specific relative primers. The control measure condition of which achieving the outgrowth of *B. cereus* spore and reducing level of AFB1 was conducted under sauerkraut parteurization.

The second phase was focused on verification of cross-preventive control measure condition for monitoring the outgrowth of *B. cereus* spore and AFB1 reduction level during sauerkraut fermentation. The verification of pasteurization control on *B.*

cereus spore and AFB1 hazard safety was also evaluated.

1.5 Expected results

To assure the cross-preventive stress condition of sauerkraut process, NaCl 2%, lactic acid 1.2%, temp 82°C for 20 min on specific resistant high risk hazards of *B. cereus* spore targets and AFB1 was gained. In addition, to get the scientific data from factors control parameters, sauerkraut condition appling in predictive microbiological modelling assess risk due to the survival, by ensuring low initial contamination levels in sauerkraut and by preventing outgrowth of *B. cereus* spore and aflatoxin B1 level reduction under significant sauerkraut stress conditions for being documentary data implied in the development of Food Safety Management Systems (FSMSs).

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CHAPTER II

LITERATURE REVIEWS

2.1 Lactic acid fermentation of sauerkraut

Lactic acid vegetable fermentation represents the oldest method of food preservation which enhances the daily consumption of vegetable products where lowing income in a small-scale production in developing countries (Motarjemi, 2000). The advantage of the variety fermented vegetable products is reflected on not only the digestibility improvement, but also in increasing nutritive value; extending seasonal availability, overall acceptable but prolonging shelf life and providing food safety (FAO, 1997; Bell et al., 2017; Karovicova, 2003; Cagno et al., 2013; Demir et al., 2006; Wuytsa, 2018; Moon et al., 2018, Hong et al., 2014; Nguyen et al., 2013; Xiong, Guan, Song, Hao, and Xie, 2012; Xia et al., 2017).

A large number of lactic acid fermentated vegetable has been studied extensively to determine the microbiological and biochemical changes occurring during fermentation process (Breidt et al., 2013). For examples, fermentation of cabbage, cucumber, carrot, beets, turnips, green beans, sliced green tomatoes, olives, cauliflower and the others. Most of them are carried out by lactic acid bacteria (LAB) like Lactobacillus plantarum, Leuconostoc mesentroides, and Lactobacillus brevis (Swain et al., 2014). Besides, others genus including *Enterococcus, Vibrio, Weissella, Pediococcus, Enterobacter, Salinivibrio, Acinetobacter, Macrococcus, Kluyvera* and *Clostridium*, also were explored (Wood, 1998). During fermentation, *L. mesenteroides*

grows rapidly and produces carbon dioxide and organic acids that cause lower pH (pH<7), thereby inhibiting the development of undesirable microorganisms and the activity of their enzymes as well as preventing unfavorable softening of the vegetables (Du, 2018; Wuytsa, 2018; Moon et al., 2018; Baráth et al., 2004; Maifreni et al., 2004; Rhee et al., 2011) as the Figure 2.1.

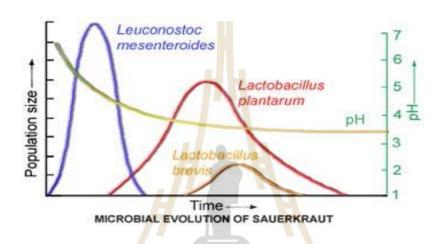


Figure 2.1 Evolution of microorganism during typical sauerkraut fermentation.

(Cano and Colome, 1986)

Nowadays, only sauerkraut, cucumber, olives, and kimchi are produced in industrial scale and increase their safety, nutritional, sensory and shelf-life properties through lactic acid fermentation under standardized industrial conditions. Many other vegetables and fruits, such as carrots, cherries, eggplants, green tomatoes, pineapples, and sweet peppers are used for fermentations on household or small scale (Cagno et al., 2013). Also, microbial cultures can be used to produce several compounds such as enzymes, flavors, fragrances etc as shown in Table 2.1.

Table 2.1 Examples of acid-fermented vegetables produced in different regions of the world.

Product	Country	Major	Microorganism	Usage	
name	·	ingredients	8	0	
Sauerkraut	Germany	Cabbage, salt	Leusconostoc	Salad, side dish	
			mesenteroides,		
			Lactobacillus		
			plantarum		
Kim Chi	Korea	Korean cabbage,	L. mesenteroides, Lb.	Salad, side dish	
		radish, various	brevis, Lb.		
		vegetable, salt	plantarum		
Dhamuoi	Vietnam	Cabbage,	L.mesenteroides, Lb.	Salad, side dish	
		various	plantarum		
		vegetable, salt			
Dakguadong	Thailand	Mustard leaf,	Lb. plantarum	Salad, side dish	
	5	salt	165		
Burong	Philippines	Mustard	Lb.bevis,	Salad, side dish	
mustasa		ร เสยเทคเเ	peuiococuscerevisia		

(Rhee et al., 2011; Breidt et al., 2013).

However, lack of knowledge and understanding toward fermented food preparation may limit their usage, in term of Food Safety Management System, Hazard Analysis and Critical Control Point (HACCP) studies in Africa illustrated that some fermented products may pose a safety risk due to the process and the hygienic conditions

during preparation (Mugula et al., 2001), which concerning pathogenic bacteria or chemical intoxicants produced by contaminating microorganisms, yeasts or moulds in fermented foods. However, this knowleage is still poorly understood, so that concerns about food safety are raised. Therefore, an understanding of microorganisms for lactic acid fermentation at a molecular level is still required to develop the product with high nutritional value.

2.2 Microorganisms play the important roles of sauerkraut fermentation

Sauerkraut produced from *Brassica* vegetables is a worldwide natural fermented vegetable, both traditionally and commercially, by which lactic acid fermentation of shredded and salted white cabbage, based on spontaneous fermentation of various LAB present in raw material or starter cultures used (Kalac, Spicka, Marti, and Pelikanova, 2000; Hunaefi et al., 2013). *Leuconostoc (Lc.)* and *Lactobacillus (L.)* species, particularly *L. plantarum* and *Lc.mesenteroides*, are the predominant microflora as mixed starter cultures during sauerkraut fermentation (Jagannath, Raju, and Bawa, 2012; Hunaefi et al., 2013; Peñas et al., 2010). Hetero-lactic fermentative *Leuconostoc* species are found at the beginning of the fermentation while homo-lactic fermentative *Lactobacillus* and *Pediococcus* species are presented at the end of the fermentation process as shown in Figure 2.2 (Jagannath et al., 2012; Mazzoli, 2014; Xiong et al., 2016).

Thin arrows stand for single enzymatic reactions, while thick arrows stand for multiple reaction pathways, AlaDH, alanine dehydrogenase; ALS, α-acetolactate synthase; EPS, exopolysaccharides; GAP, glyceraldehyde-3-phosphate; LDH, lactate dehydrogenase;

PDH, pyruvate dehydrogenase; PFL, pyruvate-formiate lyase; PHB, polyhydroxybutyrate (Adapt from Mazzoli, 2014).

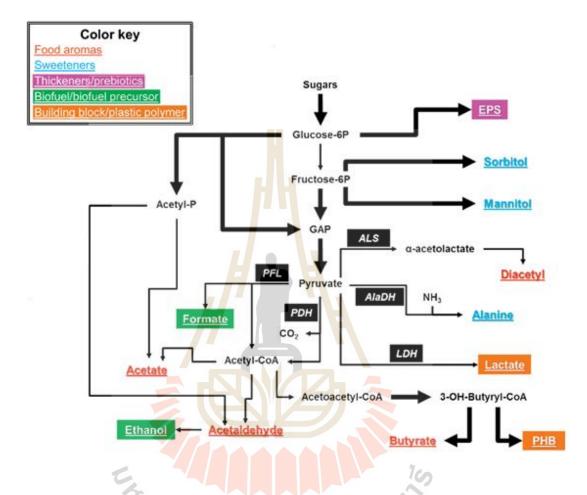


Figure 2.2 Schematic overview of the metabolic pathways enabling LAB to produce some of the most industrially desired molecules by sugar fermentation (Mazzoli, 2014).

Those metabolites such as lactic, acetic, butyric, and propionic acids are produced during fermentation, as well as other metabolic end-products (antioxidant, bacteriocins, sugar, etc) contribute to the sensory chatacteristics and bio-preservatives which lead to the food quality and the microbiological safety assurance (Holzapfel, 1995). Naturally, preservatives in foods such as lactic and

acetic acid cause the lower level of pH which penetrates into the cell membrane. Such the stress acidic, proton leakage are faster than the cell's ability to maintain homeostasis (Krulwich et al., 2011). Organic acids penetrate the cell membrane and after dissociation inside the cell, the released proton acidifies intracellular pH, both result in intracellular acidification to levels that damage or disrupt key biochemical processes, the membrane-impermeable ionized form of the organic acid accumulates and the constant influx of protons will eventually deplete cellular energy, causing cell death in enterobacteriaceae (Jarboe et al., 2013). Furthermore, brine condition induces osmotic pressure and decreased water activity. Under normal conditions, bacterial cells maintain turgor pressure, which is a higher internal osmotic pressure than that of the surrounding medium. As a result, pressure was exerted outwards on the cell wall, transporting protons in a pH-driven process inside the cell, eventually leading to disruption of the proton motive force and this turgor pressure was the mechanical force for cell elongation (Csonka, 1989; Munder et al., 2016). When the osmotic pressure in the surrounding environment increased, cells activated osmoregulation systems to prevent shrinkage and eventual plasmolysis. Osmotic stress conditions mimicked other physiological features of starvation (Matin et al., 1989; Karas et al., 2015). In addition, increased osmolality in bacterial cells was associated with the inhibition of DNA replication, cell growth, and nutrient uptake (Csonka 1989; Karas et al., 2015).

However, food fermentation product is not completely free of microbiological, a huge diversity of microorganisms is associated with the enormous variety. In this wide microbio-diversity, it is possible that the presence of microbial pathogens (*Salmonella*, enterohaemorrhagic Escherichia coli, Campylobacter, Listeria, Shigella, Yersinia), parasites (*Cryptosporidium*, *Cyclospora*, helminths) and viruses (hepatitis A,

noroviruses) (FAO/WHO, 2008) and toxic by-products of microbial origin, including mycotoxins, ethyl carbamate and biogenic amines, are aspects liable to reduce the food safety (Capozz et al., 2017). However, total 68 fermented vegetables samples were collected randomly from local market in Phnom Penh, Campodia. The containinatation of spoilage and pathogenic bacterial was showed in Table 2.2 and Table 2.3 (Chrun, 2017).

Table 2.2 Isolated bacterial species from total 68 fermented vegetable samples in local makets in Phnom Penh, Cambodia 2017.

Gram negative bacteria	Gram positive bacteria					
Citrobacter freundii	Enterococcus faecium					
Cronobacter sakazakii	Enterococcus durans					
Ewingella americana	Enterococcus faecalis					
Enterobacter cloacae	Aerococcus urinae					
Escherichia. coli	Staphylococcus lentus					
Klebsiella pneumoniae	Staphylococcus xylosus					
Moellarella wisconsensis	Listeria grayi					
Rahnella aquatilis	Bacillus cereus					
Serratia plymuthica						
Aeromonas salmonicida						
Rhizobium radiobacter						
Shewanella putrefaciens group						
(Chrun, 2017).						

Table 2.3 Comparison of contamination rate of bacterial strains between fermented single-type and fermented mixed vegetables.

	Fermented singl	e-type	Fermented mixed	d	P-value of
Bacterial strains	vegetables		vegetables		odds ratio
	Positive / Total	(%)	Positive / Total	(%)	_ ouus rano
Coliform bacteria	6 / 48	13	10 / 20	50*	*0.05
Escherichia coli	3 / 48	6	4 / 20	20	0.11
Cronobacter sakazakii	1 / 48	2	0 / 20	0	0.88
Opportunistic	6 / 48	13	1 / 20	5	0.40
non-Enterobacteriaceae					
Enterococcus spp.	13/48	27	10 / 20	50	0.10
Staphylococcus spp.	4 / 48	8	0 / 20	0	0.35
Listeria spp.	3 / 48	6	1 / 20	5	0.84
Bacillus spp.	17 / 48	35	4 / 20	20	0.22

The superscript * represents a statistically significant difference in the detection rate (P<0.05) between fermented single vegetable and fermented mixed vegetables (Chrun, 2017).

However, fermentation parameters are sometimes very difficult to control, it depends on the kind of raw materials and initial load of contamination levels of hygiene and sanitation, fermentative behavior, the degree of acidity achieved and obtained products. These factors also are hardly controlled to the household condition. Consequently, fermentation can result in undesirable products that are sometimes even risky or dangerous (Motarjemi, 2002).

The botanical, physical, chemical and textural properties of the various fruits and vegetables differ widely, causing several differences of the applied technology. Since it

is not possible to discuss the individual working steps in detail, a highly simplified flow sheet for manufacturing lactic acid fermented vegetables is given in Figure 2.3.

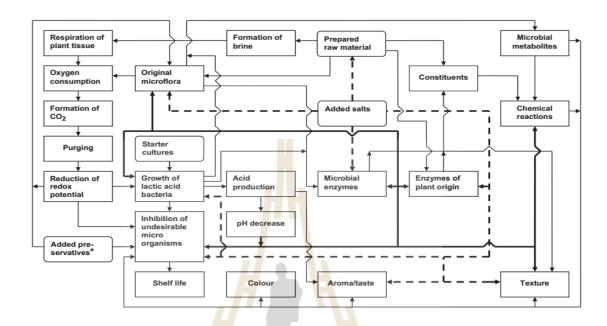


Figure 2.3 Significant reactions and their most important interactions that take place during the lactic acid fermentation of cabbage to sauerkraut. The bold dotted lines indicate the reactions influenced by the added salt. The bold solid lines indicate the influence of lowering the pH caused by the microbial production of acids on the entire process. Only permitted in a few countries (Buckenhueskes, 2015).

2.3 Risk hazards associated with sauerkraut

The only fermentation presentation technique could impossibly eliminate all the health risk in finish product since food harmful pathogens e.g. bacterial, fungi, parasite and some virus, might be survivors. The microbiological food safety control is seriously importance for all the companies and government organizations of which are involved in food chain production, particularly processing, distribution, retail, correspondingly to

regulation of foods and drinks. For the customer protection, the good manufacturing and hygiene practice (GMP/GHP) followed by the process control based on the HACCP principles should be applied as preventive measures, whereas the qualitative risk assessment will be a scientific information for the description treatment in order to estimate the magnitude of risk and the impact of factors affecting risk.

Sauerkraut quality control depends on the precise sanitary practices implied in the fruit and vegetable industry, regarded as safe products arising from the physico-chemical characteristics attained during the process (i.e., pH, acidity, salt content). At present, the process depends almost on the restricted microorganisms present in the raw material and processing environment starting at farm level by the use of unsafe water contaminated with faecal matter, or contaminated manure and access of livestock or wild animals in the field lead to the microbial spoilage and pathogen (Faour-Klingbeil, Murtada, Kuri, and Todd, 2016).

The environmental process factors are great consequence for the consideration to do the plan for microbiological quality control of fermented vegetable. Those of the key factors are such as vegetable, water, brine, pipes, containers, equipment, and atmosphere. According to Bredt 2000, microbial populations of fresh fruits and vegetables range from 10² to 10⁸ CFU/g or higher. Indistinguishably, high microbial loads are existing in cabbage resulted in the short period of the fermented cabbage shelf-life, dealing with cross-contamination from food pathogens, such as *E. coli, Samonella* (Harris, 2003; Mukherjee et al., 2007), *L. monocytogenes* (Harris, 2003; Prazak, 2002; Amoah et al., 2006; Erickson, 2010), *B. cereus* (Harris, 2003; Erickson, 2010), *Clostridium botulinum* (Harris, 2003; Soriano 2000) which tolerate high acidity environments and heat tolerant as well. Vegetative bacterial pathogens can present on fresh produce and ingredients that are used in the manufacture of fermented and

acidified foods (Beuchat, 2002; Erickson, 2010; Kim, 2004).

Ascospores of heat-resistant molds can often survive commercial pasteurization heat treatments usually applied to fruits and fruit products (Salomão et al., 2007). Most yeasts and molds are heat-sensitive and destroyed by heat treatments at temperatures of 140-160°F (60-71°C). Some molds make heat-resistant spores, however, it can survive under heat treatments in pickled vegetable products. When jars or containers of thermally processed acidified vegetables are improperly sealed or cracked and let oxygen get in. Under these conditions, heat resisted spores treatment may germinate and grow on the liquid surface at where air is present. Heat-resistant molds process ascospores or similar structures are mostly characterized as heat resistance, resulting in visible growth of mycelia sauerkraut products. As Atrih 2001 demonstrated that survival curves of spores prepared in the CCY and NB media and then subjected to heat at 85°C, showed 48.5% and 0.3% survival after 60 min at 85°C, respectively. Nevertheless, adding MnCl₂ (0.01 mmol 1⁻¹) to NB resulted in a seven fold gain in spore survival. Whilst, mycotoxins which represent the major of chemical risk hazard of traditional vegetable products, it was found that the major increase in heat resistance of spores prepared in NB supplemented with CCY salts compared with that supplemented with Mn²⁺ is of interest, especially as the addition of CCY salts only results in an alteration of Mn²⁺ content. The presence of salts at high concentration in the medium certainly contributes to spore resistance. However, availability in an appropriate ratio is probably important for the attainment of optimal spore properties (Slepecky and Foster 1959). High salt concentrations together with heat treatment molds may also generate a more homogeneous spore population and produce various mycotoxins during their growth in fruit products. The main characteristics of the *B. cereus* are summarized in Table 2.5.

The high risk hazard microbiological on pickle product is thermophilic spore

forming bacteria. Its sources come from soil, water and in the gastro-intestinal tract of eukaryotic organisms (Jensen, Hansen, Eilenberg, and Mahillon, 2003; Swiecicka, 2008) and can be introduced into the pickle environment from various sources during production, handling and processing, mainly from cleaned and sanitized equipments (Valero, 2002; Roberts et al., 1982; Harmon et al., 1987; Magnusson et al., 1990; King et al., 1991; Harmon and Kautter, 1991; Kaneko et al., 1999).

Table 2.4 Characteristics of *B. cereus* resistance and growth.

Characteristics	Value
Spore heat resistance D_{100}	1.2-8 min
Spore heat resistance z	6-9°C
Germination temperatures - limits	5-50°C
Germination temperatures - optimum	30-35°C
Growth limits pH	4.3-9.3
Growth limits temperatures	5/15-35/50°C
Growth limits $A_{\rm W}$, broth	0.95
Generation times, broth culture 35°C	ulagas ^{ul} ca 20min
(McClure, 2002).	

Cabbage is contaminated *Bacillus* spores as a result of processing or post processing contamination (Erickson, 2010; Harris, 2003). *Bacillus* species have been associated with the spoilage of sauerkraut as well as food safety incidents (Dierick et al., 2005). The presence of spore forming pathogenic *Bacillus* species, such as *B. cereus*, is frequently the cause of several foodborne illnesses. The

effective dose for the emetic syndrome is 10^5 - 10^8 cells/g of food where the toxin is pre-formed in the food, and 10^5 - 10^7 total cells to infect the small intestine for the diarrheagenic syndrome (Table 2.4). For either intoxication, symptoms resolve after 6-24 hours, and while some patients may be sufficiently severely affected to be hospitalized, reports of mortality are extremely rare. Recovery is usually complete in 24-48 hours, and no specific treatment other than replacement of fluid and salts loss is indicated.

"5/35 °C limits for psychrotrophic strains; 15/50°C for mesophilic strains.

Spore germination may occur up to about 60°C" (Adapt from McClure, 2002).

Table 2.5 Characteristics of *B. cereus* food-poisoning syndromes.

Characteristic	Emetic syndrome	Diarrhea syndrome		
"Infective" dose	10 ⁵ -10 ⁸ /g food	10^5 - 10^7 total cells		
		consumed		
Production site of toxin	in food	small intestine		
Symptoms	nausea and vomiting	abdominal pain, diarrhea;		
475	possibly also diarrhea	occasionally nausea		
Incubation time	1-5 hours	8-16 hours		
Duration of symptoms	6-24 hours	12-24 hours		
Toxin characteristics	small peptide; heat	at least three		
	resistant; acid stable;	proteinenterotoxins		
	hydrophobic; protease	recognized; heat and		
	stable; non-immunogenic	protease sensitive;		
		immunogenic		

(McClure, 2002).

Moreover, it is a great safety concern for fermentation vegetable industry as it is associated with incidences of food poisoning by producing enterotoxin. *B. cereus* comprises the largest group of endospore forming bacteria and is now attracting a great interest among researchers as the members are not only associated with foodborne outbreaks but also responsible for spoilage of food products.

The hazard mycotoxins are fungal secondary metabolites which have various acute and chronic effects on human health, therefore, they represent another distinguish risk associated with sauerkraut product consumption. Moreover, as a recommend to European Chilled Food Federation (2006), Table 2.5 shows these pathogens need to be taken into consideration. Commonly accepted growth boundaries of pathogenic microorganisms.

In addition, the contaminated mycotoxin product is currently problem in the world. There is no doubt that lactic acid fermentation and its products have gained the benefits and may be considered in food safety. However, mycotoxins and microbial spores such as those associated with *Aspergillus flavus*, *Aspergillus oryzae*, *Penicillium roqueforti* and other fungal toxins, such as those associated with AFB1, are known to be quite stable at high dose of temperature. Major risk containminated high risk hazards are the use of contaminated raw materials, lack of pasteurization, and use of poorly controlled natural fermentations. Also suboptimal fermentation starters and inadequate storage and maturation conditions as well as consumption without prior cooking may reduce the safety of fermented foods. Table 2.6 shows the occurrence of different aflatoxins in various fermented foods.

Table 2.6 Description of the occurrence of different aflatoxins in various fermented foods.

	A 011	G 4	Detected	D / // N // 1	D.C.	
Food	Aflatoxin	Country	Level	Detection Method	References	
White dent	Aflatoxin	Nigeria	1.05-2.55	Spectrophotometry	Adegoke et al. (2010)	
maize (Zea	B1		$\mu g/ml$			
mays)						
Beer and	Aflatoxin	Germany	43 ppb	Photon-induced	Rasch et al. (2010)	
wine	B1			fluorescence		
Fermented	Aflatoxin	ThaiLand	0.3- <mark>2.15 μg/kg</mark>	ELISA	Charoenpornsook and	
alcoholic	B1				Kavisarasai (2014)	
beverages						
Blue cheese	Aflatoxin	ThaiLand	0.5-1.25 μg/kg	ELISA	Charoenpornsook and	
	B1		/ . \ .		Kavisarasai (2014)	
Fermented	Aflatoxin	ThaiLand	0.2-3.20 μg/kg	ELISA	Charoenpornsook and	
soybean	B1				Kavisarasai (2014)	
product						
Finish red	AFB1	France	0.012-0.126	Reversed-phase	Khoury et al. (2006)	
wine (grapes)			μg/L	HPLC		
Traditional	Total	Korea	0.00-42.2	ELISA	Kim and Kim (2012)	
Korean	aflatoxin		μg/kg			
Doenjang						
Cheese	Aflatoxin	Slovenia	50 μg/kg	ELISA	Torkar and Vengust	
	M1			169	(2008)	
Lighvan	Aflatoxin	Iran	0.85 μg/kg	TLC	Fallah (2010)	
cheese	M1	ายาลัง	un oful	ลยีสร		
Traditional	Aflatoxin	Iran	0.018 µg/kg	TLC	Fallah (2010)	
Iranian	M1					
yogurt						
Commercial	Aflatoxin	Iran	0.038 µg/kg	TLC	Fallah (2010)	
Iranian	M1					
yogurt						

(Frias, Villauenga and Peñas, 2017).

According to the United States Department of Agriculture (USDA) and all the world, the maximum level for Aflatoxin B1 on food is 5μg/kg and the maximum tolerated levels (MTLs) authorities have been legislated by national and international (FAO, 2004; WHO 2006; Wu, 2013). However, in developing countries where food safety compliance can be low and significant levels of the food supply are locally consumed by the producers or purchased at local markets, more comprehensive strategies are required. In view with its toxic nature, Food and Drug Administration (FDA) has set the limiting value for its presence at 20 μg/kg for all foods and 300 μg/kg in feed (USFDA, 2009). In this regard, risk assessment is considering tool in dealing with food safety issues. In order to prevent mycotoxin contamination of a fermented food, it is necessary to use a mycotoxin-free raw material and to prepare it in good sanitary and hygiene conditions (Adekoya et al., 2017; Inetianbor, 2017).

2.4 Cross-preventive stress responses on high risk hazards in sauerkraut process

Various factors influence the rate at which microorganisms grow in foods. These include the intrinsic properties of the foods (nutrient content, pH, redox potential, water activity, etc.) as well as the extrinsic factors related to the conditions under which they are stored, e.g., temperature and relative humidity. Implicit factors are the results of mutual interactions in mixed microbial populations as a Table 2.7.

Table 2.7 The following factors contribute to the growth of microorganism in foods.

Intrinsic factors	Extrinsic factors	Implicit factors	
Nutrient content, pH	Temperature	Synergism	
Redox potential and oxygen	Related humidity	Antagonism, Commensalism	
Water activity	Gaseous	Growth rate	
Microflora	environment		
	Atsmosphere		

(Bhat, Alias and Paliyath, 2012).

To reduce or prevent microbial spoilage of foods, four basic principles can be applied: (1) minimize the level of microbial contamination; (2) inhibit the growth of the contaminating microflora (3) kill the contaminating microorganisms; and (4) remove the contaminating microorganisms. The fermentation involves a combination of the first three principles, and is achieved by creating an environment for the growth of specific microorganisms that can impart desirable taste, flavor, texture, or appearance to foods (Guizani and Mothershaw, 2007).

Exposing microorganisms to a stress may induce adaptation to subsequent lethal levels of the some stress condition. This phenomenon is referred to as stress adaptation. Microbial adaptation to a stress may also enhance cell tolerance to multiple lethal stresses known as cross-protection. Foodborne pathogens and other microorganisms are often exposed to stresses that cross-protect them against the lethality of various preservation factors (Table 2.8). Therefore, cross-protective responses are the reaction of pathogen when evaluating the efficacy of intervention strategies to achieve food safety and to preserve the quality of food products.

Table 2.8 Examples of adaptive responses leading to cross-prventive in microorganisms previously exposed to sublethal stress.

Sublethal	Cr	oss-pr	otecti	ve resp	onse t	to letha	al stres	ses		
stresses	Heat	Cold shock	Freezing	Freeze-drying	Osmotic	Acid	Ethanol	Oxidative	Microorganism	References
			+						Lactobacillus	Walker et
Heat			+	+	ľ	H			johnsonni Lactobacillus lactis	al. (1999) Broadbent and Lin (1999)
						+			B. cereus	Browne and Dowds (2002)
Acid			+			7	+	+	Lactobacillus acidophilus	Lorca and Valdez (2001)
reid	+						+		Bacillus subtilis	Browne and Dowds (2002)
	+	37:	ne	4				45	Lactobacillus paracasei	Desmond et al. (2001)
	+		311	la	UIN	คโบ	lag	10,	Lb. acidophilus	Kim et al. (2001)
Osmotic	+					+	+	+	B. cereus	Browne and Dowds (2001, 2002)
	+								B. cereus	Periago et al. (2002b)

(Griffiths, 2005).

B. cereus is a spore forming, Gram-positive bacterium and cause of foodborne poisoning. Its spores resist the heat, dry conditions, a wide range of sanitation procedures and food processing treatments. The major cause of the food safety are B. cereus spores can be possibly survive at the lowest level along the food production be multiplied in foodstuffs to reach hazardous levels before food consumption. B. cereus is involved not only in foodborne poisoning but also in various severe clinical human infections, septicaemia, and meningitis, gingival and ocular infections. Cereulide is not easily destroyed by heat treatments. For instance, it can resist 90 min at 126°C (ICMSF, 1996) and is also resistant to acid conditions. Cereulide will therefore not be eliminated from foods in which it has been produced.

2.5 Hazards characterization of high risk significant microorganisms in sauerkraut

2.5.1 Bacillus cereus

When food are contaminated with bacterial spore, they are difficult to destroy since spores displays high adaptive capacity towards different environmental stress conditions including UV radiation, heat, and other stresses often applied during food production and preservation to making it a survial (Nicholson, 2000; Setlow, 2006; Moeller et al., 2014 Eijlander et al., 2014). *B. cereus* is a Gram positive rod-shaped bacterium, able to form endospores under strick conditions and ubiquitously present soil and, plant rhizosphere, intestinal tract of insects and animals, and it is impossible to prevent contamination at the primary production level. The bacterial species *B. cereus* accounts for 1.4%-12% of foodborne illness outbreaks worldwide.

Heat treatments are conventionally applied in food processing to reduce the

microbial load of food products, however, consumer preferences are on a rise towards traditional food products with higher sensory and nutrient value, which are heat process may be become milder. *B. cereus* forms endospores that are heat resistant to pasteurization but the vegetative cells are rapidly killed at 65°C (Holsinger et al, 1997). *B. cereus* endospores are partially resistant to food processing for example gamma radiation, dehydration, pasteurization, and other physical stresses, and they have ability to promote biofilm forming capability on a variety of substrates. Bacterial biofilm productions are similar regulation as toxins and other extracellular virulence determinants (Alyssa, 2018).

Competing microflora is subjected to be eliminated, whereas endospores still survive after pasteurization or heating. On the orther hand, during food cooling, endospores germinate and vegetative cells proliferate, producing several toxins, one of which is highly resistant to heat and to pH between 2 and 11. The infection may be almost diarrhoeal, but some cases described nausea and vomiting (Ehling-Schulz et al., 2004; Schoeni and Wong, 2005; Roy et al., 2007; Ouoba et al., 2008), a number of food borne outbreaks in Europe 18 (Wells-Bennik, 2016). *B. cereus, Clostridium perfringens and Clostridium botulinum* are traditionally pathogenic group associated with *B. cereus* spore.

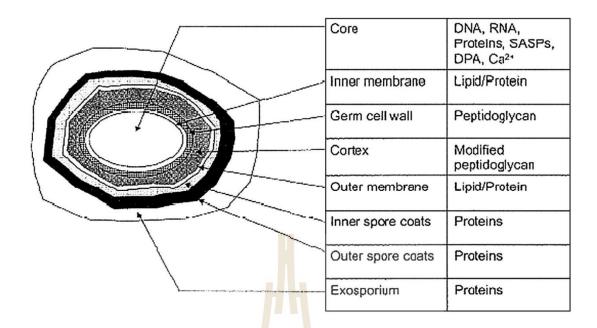


Figure 2.4 Structure of *B. cereus* spore.

(Adapt from http://www.onlinebiologynotes.com/bacterial-spore- structure-types-sporulation-germination/).

B. cereus is frequently associated with various types of foods; rice, vegetable, meat, milk, and dairy product (Andersson, 1995; Choeni, 2005). Spore forming *B. cereus* composed of two small core molecules which response risk assistant the survival. The first is the water; it comprises 75-80% of the protoplasm wet weight of a cell growing with a comparison of spores, only 27-55% of the core, depending on the species. The low moisture content of the core probably affects enzyme inactivation influenced the moist heat resistance of the spores. The second important molecule in the core is the pyridine-2, 6-dicarboxylic acid (dipicolinic acid, DPA), DPA consisting 5-15% of the dry weight spores and contributes to the inactivated state and dehydration core. Besides, the small acid soluble proteins protect the DNA spore from damage caused by ultraviolet radiation, heat and some chemicals genotoxic (Silva, 2013). There

are some proteins which involve in protection of macromolecules, such as and *Gro*ESL, *Dna*K, and enzymes that produce alkaline compounds, such as and arginine deiminase, the ammonium-forming enzymes urease (Mols et al., 2010).

According to the report of EFSA 2005, the hight mount of *B. cereus* (10⁵-10⁸ CFU/g) was found in relevant food safety product due to equipment failure (Andersson, 1995). In 1930, a cheese spoilage study led to isolate *B. cereus* ATCC 10987, this strain could produce high biofilm forming capacity because of bacterial cell-cell signalling, or quorum sensing (Hayrapetyan et al., 201; Tasneem, 2018). A biofilm, is a complex multicellular structure in which cells are protected by matrix components from cleaning and disinfection procedures. Eventual dispersal of the cells or spores from the biofilm into the production line may lead to contamination or recontamination of the food products.

2.5.2 Aflatoxin B1

As regard to spore of fungi, mold heat resistance is attributed to the formation of sexual spores, ascospores. Ascospores have a many kind of heat resistance, depending its characteristics for example: species, strain, age of microorganism, heating medium, pH, the presence of sugars, fats, and acids in heating medium, growth conditions, etc. The mechanisms of thermoresistance are not clear, probably because of some critical stable compounds of the germination and outgrowth is present in the heat-resistant ascospores which can produce many kind toxic secondary metabolites being the most high risk hazards such as genotoxic and carcinogenic aflatoxins B1. It is produced by both *A. flavus* and *Aspergillus parasiticus* whichever maximum level for Aflatoxin B1 on food is 5µg/kg. For the disaccharide trehalose is a major component of fungal spores and is released upon germination which trehalose has been shown to accumulate in dispersal and survival structures such as spores because it against thermal stress and dehydration

(Svanström, 2014). Aflatoxins are secondary metabolites of the fungi *Aspergillus flavus* and *A. parasiticus*, occur widely on many staple foods and cause a broad range of detrimental health effects in animals and humans (Eaton and Gallagher, 1994 as cited in Zinedine, 2009). As a consequence, maximum tolerated levels (MTLs) authorities have been legislated by national and international food standard (FAO, 2004; WHO, 2006; Wu et al., 2013). However, in developing countries where food safety compliance can be lowering and significant levels of the food supply of what locally consumed by purchased at local markets, that why more comprehensive strategies are required. In term of its toxic nature, Food and Drug Administration (FDA) has set the limiting value for its presence at 20 μg/kg for all foods and 300 μg/kg in feed (USFDA, 2009).

2.6 Food safety of fermented vegetable condition and pasteurization

Based on the Food safety management system, bacterial spores are focused to prevent at beginning step in the food chain to achieve in good practice due to the present of bacterial spore in the environment and display to resistant toward stress conditions for survival. Traditionally, applying hight temperature as sterilization processes have been performed to target heat resistant spores, aiming for production of commercially sterile foods with extended shelf life at ambient room conditions. Follow by sterilised canned foods (e.g. meat, fish, and vegetables) and ultra-high temperature (UHT) treated liquids (juices and milk) (Wells-Bennik, 2016). Consequently, milder processing techniques like pasteurization are applied often including an inactivation treatment (e.g. heat) to reduce the level microbial loads. Multiple combination factors are commonly used to control growth of microbes throughout shelf life. Control measures include intrinsic foods factors such as water activity, pH, presence of organic acids, salt including storage

and packaging conditions, such as temperature and oxygen limitations in e.g. MAP-packaging. Moreover, consumers as well as governments attemp to reducte in use of sugar and/or salt which are traditionally applied food preservatives (Pasha et al., 2014). Increasing complexity of applied strategies poses new challenges in assuring the stability and safety of a variety of foods, and particularly in predicting combined effects of individual treatments.

Sauerkraut provides essential vitamins, minerals and dietary fiber for health. A range of varierty phytochemicals commonly found in vegetables, such as flavonoids, carotenoids, and phenol which reduce the risk for various types of cancer, heart disease, diverticulosis, diabetes, stroke, birth defects, hypertension, cataracts, and obesity. However, sauerkraut is highly perishable, they need efficient and appropriate preservation technology to prolong their shelf life while maintaining nutritional value and sensory quality.

With the increased public awareness of healthy diet, thermal pasteurization has regained our attention as an effective vegetable preservation method to provide safe convenient foods with high nutrients and good sensory quality. The word "pasteurization" was originally named after the French scientist Louis Pasteur who invented the process of heating food, such as wine at 55°C for several minutes to kill disease causing micro-organisms (pathogens). Thus, traditional pasteurization refers to a heat treatment of food (usually below 100°C) to destroy all organisms dangerous to health, or a heat treatment which destroys part, but not all microorganism that cause food spoilage or that interfere with a desirable fermentation. Unlike sterilization, pasteurization process doesn't kill all the micro-organisms in the foods, only destroying the vegetative pathogenic bacteria and lowering the level of spoilage organisms that will grow under refrigerated storage.

In recent years, the development of emerging technologies which can satisfy the goals of pasteurization, calls for a broadening of the definition of pasteurization. Therefore, the National (NACMCF, 2006) has determined the requisite scientific parameters for establishing equivalent alternative methods of pasteurization, and defined it as below (NACMCF) "Any process, treatment, or combination therefore, that is applied to food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risks under normal conditions of distribution and storage".

This definition allows application of a broad range of technologies (one or in combination) to different food-pathogens for pasteurization such as ohmic heating, microwave heating, steam and hot water heating, pulsed electric field, chemical treatments, filtration, infrared, and high voltage are discharged. In addition to the processing methods, three major points are made in this definition for developing a pasteurization process:

- 1. Determining the most resistant microorganism of public health concern for the food.
- 2. Assess the level of inactivation of target microorganism needed and validate it, to make sure "not likely to present a public health risk".
 - 3. Consider the distribution and storage conditions, normally refrigerated.

According to the recommendations of NACMCF, more factors need to be considered for establishing a successful pasteurization process, such as the impact of food matrix on pathogens survival, developing specific Hazards Analysis Critical Control Point (HACCP) system and Good Manufacturing Practices (GMPs) for the process, etc.

Table 2.9 The accepted level growth boundaries of pathogenic microorganisms.

Microorganism and growth	Min temp	Min	N/2	Aerobic /
boundaries	(°C)	pН	Min a _w	anaerobic
L. monocytogenes	-0.4	4.3	0.92	Facultative
B. cereus	4	4.5	0.93	Facultative
Campylobacter jejuni	32	4.9	0.99	Microaerophilic
Cl. botulinum Mesophilic/proteolytic	10-12	4.6	0.93	Anaerobic
Cl. botulinum Psychrotrophic/non-	3.3	5.0	0.97	Anaerobic
proteolytic				
Cl. perfringens	12	5.5-5.8	0.935	Anaerobic
E. coli	7-8	4.4	0.95	Facultative
E. coli O157:H7	6.5	4.5	0.95	Facultative
Salmonella	6	4.0	0.94	Facultative
Staphylococcus aureus	5.2	4.5	0.86	Facultative
V. cholerae	10	5.0	0.97	Facultative
V. parahaemolyticus	5	4.8	0.94	Facultative
Y. enterocolitica	-1.3	4.2	0.96	Facultative

(European Chilled Food Federation, 2006).

Aflatoxins were quite stable up to their melting point of around 268°C (Arzandeh and Jinap, 2011). The affectsing aflatoxins on food producing are depend on upon the moisture content, type of food matrix (Scott, 1984). Food processing has been reported to destroy the aflatoxin to some extent while a general resistance to destruction was observed (Mobeen, Asif, and Zuzzer, 2011). Other process step for exempale, such as roasting and extrusion cooking at temperatures above 150°C, steeping were reported to be very effective

against aflatoxin (Bullerman and Bianchini, 2007). Dry heating has been reported to not very effective while heating moist meal or autoclaving groundnuts has been found to reduce aflatoxin content (Rustom, 1993; Mobeen et al., 2011). Mycotoxin concentrations were reported to reduce on aqueous cooking and steeping. Rustom (1993) reported that aflatoxins were sensitive to moist heat and the moisture content of the contaminated food was the critical factors for the destruction of toxins by heat.

The varying degrees of thermal degradation of different mycotoxins have been reported under elevated temperatures depending on the degree of heat penetration, temperature, exposure time, moisture content, pH of food and the concentration of the mycotoxins in the food matrix (Bozo Ilu, 2009; Kamil and Lupuliasa, 2011; Meca et al., 2010; Velazhahan et al., 2010).

Processes applied on foods such as heating, roasting and baking can reduce the levels of aflatoxins but do not completely eliminate the toxins, and the degree of elimination is variable and depends on the process as well as the conditions under which the process is applied (FAO/WHO, 2017).

In term of food safety, most LAB are used for preservation of food products, by inhibiting the growth of spoilage and pathogenic bacteria through production of organic acid (lactic acid, acetic acid), bacteriocins (Nisin), diacetyl, hydrogen peroxide, alcohol, carbon dioxide, and acetaldehyde during carbohydrate (sugar, fructose) fermentation vegetable (Gálvez, 2008; De Vuyst, 2007; Albano et al., 2007). However, due to the relative abundance of mycotoxins-producing moulds in the soil, crops grown under conventional and/or organic agriculture are both not immune to mycotoxins contamination (IARC, 2007).

LABs are reported to be able to metabolize or transform mycotoxins into compounds that are less toxic. The ability of the LAB strains to repress mycotoxins by

producing low-molecular-weight metabolites (lactic acid, acetic acid and/or binding of the Aflatoxin to bacteria cell wall has been reported (Dalie, 2010). LAB can either use sorption and/or enzymatic degradation to detoxify mycotoxins by asorbing mycotoxins either by attaching them to their cell wall components or by active internalization and accumulation (Gobbetti et al., 2005). During cell rupture, it is postulated that LAB can release molecules that potentially inhibit mould growth and therefore lead to a lower accumulation of their mycotoxins (Zinedine et al., 2005). Some LAB, have been identified with the strain-specific ability to reduce mycotoxins, especially aflatoxins with great efficiency (Haskard et al., 2001). For example, an *in vitro* study on probiotic LAB strains such as *Lactobacillus rhamnosus* LC-705 and *Lactobacillus rhamnosus* GG, demonstrated that they were quite effective in aflatoxin B1 reduction, with more than 80% of the toxin removed (Shah, 1999).

In conclusion, the high risk hazard of spore forming *B. cereus* and heat stable AFB1 are the most critical biological and chemical hazards, respectively in sauerkraut. To asssure the safety of lactic acid fermented vegetable with brine (NaCl) as sauerkraut, good of thermal inactivation parameters is essential for designing pasteurization processes.

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CHAPTER III

RESEARCH METHODOLOGY

3.1 Assessment of cross-preventive condition for sub-lethal stress of

- B. cereus spore targets
- 3.1.1 B. cereus spore preparation

A pure culture of *Bacillus cereus* (*B. cereus*) ATCC 11778 was obtained from the Department of Medical Science, National Institute of Health of Thailand. The culture was maintained on tryptic soy agar slant. The strain was maintained at -20°C in tryptic soy broth with 30% (v/v) glycerol until use.

The sporulation of *B. cereus* was carried out according to the method as described earlier (Evelyn and Silva, 2015; Vercammen, Vivijs, Lurquin, and Michiels, 2012). *B. cereus* from frozen stocks was transferred in to nutrient broth (NB) and shake-flask culturing overnight at 37°C at 200 rpm in a water bath Shel lab WSBR27 (Lab Leader Co. Ltd, NY 14519 USA). Aliquots of 0.1 ml cells in NB were spread on to NA plate supplemented with MgSO₄.7H₂O (0.51 g/l), KCl (0.97 g/l), CaCl₂.2H₂O (0.2 g/l), MgCl₂.2H₂O (2.5x10⁻³ g/l), FeSO₄ 7H₂O (5.5x10⁻⁴ g/l) to induce sporulation. After 10 days of sporulation at 37°C, spores were harvested. The obtained bacterial spore suspension was centrifuged and washed three times with sterile phosphate buffer (pH 7.2) at 4000×g for 15 min at 4°C. The spores were resuspended in PBS pH 7.2, subjected to 80°C for 10 min to kill any remaining vegetative cells and immediately used for subsequent experiments (Ju et al., 2008; Wang et al., 2017).

3.1.2 *In vitro* model of multiple stress factor preparation

The collected spores were suspended in PBS pH 7.2 to a final concentration approximately 10⁸ spores ml⁻¹. In order to study the effect of multiple hurdle factor, 3³ factorial experiment NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), temperatures 65-85°C for 20 min in NB were performed and the contact time was 4 hours. After multiple stress factor treatments, all samples were kept in ice water bath or cold water at 4°C immediately prior to other parameters analysis. The spore without treatment was used as a control.

3.1.3 Monitoring of stress target change of *B. cereus* spores

3.1.3.1 Spore germination rate

To quantify the spore germination rate of *B. cereus* after treated with cross-preventive factors, one ml of each homogenized sample was serially diluted in 9 ml of sterile PBS buffered, and 0.1 ml of diluents were spread-plated on to plate count agar and incubated at 37°C for 24 h. The colonies were enumerated and calculated for the level of sub-lethally injured *B. cereus* ATCC 11778 spore. The spore germination rate for each tested sample was expressed as (1).

Spore germination rate (%) =
$$\frac{N}{N_0}$$
x100 (1)

Where: N_0 are the numbers of viable spore before treatment.

N are the numbers of viable spore after treatment.

3.1.3.2 Spore membrane permeability

The alteration in spore membrane permeability was detected by the crystal violet assay, where a higher % uptake of the dye indicates greater membrane permeability (Devi, Nisha, Sakthivel, and Pandian, 2010). The untreated and treated sample were washed twice with 10 mM PBS by centrifugation at 6700×g for 5 min at 21°C. The pellet was suspended in PBS (10 mM, pH 7.4) containing 10 mg/mL of crystal violet. The uptake of dye of spore was performed in a dark incubator at 37°C for 15 min. Then the suspensions were centrifuged at 13,000×g for 15 min and the absorbance of the free spore supernatant was measured at 590 nm using spectrophotometer (Qiumin, 2016).

The % uptake of crystal violet was calculated as the formula given below:

Uptake(%) =
$$\left(1 - \frac{\text{Abs value of supernatant}}{\text{Abs value of the crystal violet solution}}\right) \times 100$$
 (2)

3.1.3.3 Spore membrane fluidity

The spore membrane fluidity were studied by 1,6- Diphenyl-1,3,5-hexatriene (DPH) probe fluorescence. Untreated and treated samples were rehydrated and washed twice with sterile 10 mM PBS. DPH was dissolved in methanol and then diluted to 0.004 mM in PBS. The same amount of DPH molecular probe was added to a suspension of spores to a final concentration of 0.004 mM. Staining spores was incubated with DPH solution in a dark incubator at 37°C for 30 min. The samples were washed twice by centrifugation at 2700×g for 5 min and then suspended in sterile 10 mM PBS. The DPH fluorescence intensity was measured with a spectrofluorometer. The excitation and emission wavelength were 350 nm and 425 nm, respectively (Modified from Herman et al., 2015; Liu, Duan, Wang, and Li, 2010).

3.1.3.4 DPA release

The colorimetric assay (Ahn, 2007) with some modifications was used to determine the DPA of *B. cereus* spores. A 5-ml sample was centrifuged at $3,000\times g$ for 20 min to remove cell debris and 4 ml of the supernatant was mixed with 1

ml of freshly prepared reagent containing 1% ascorbic acid, 1% Fe(NH₄)₂(SO₄)₂6H₂O and in 0.5 M acetate buffer (pH 5.5). The absorbance was measured at 440 nm using a Jasco V-670 absorption spectrometer (JASCO Germany GmbH). The control sample was thermally treated at 121°C for 20 min to determine the total DPA content. An external standard curve was prepared at 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mg/ml of pyridine-2, 6-dicarboxylic acid as described for the sample.

3.1.3.5 DNA release

The release of cellular nucleic acids was detected by measuring A260 (3) (Diao, Hu, Zhang, and Xu, 2014). The samples were centrifuged at 13,000×g for 15 min at 20°C, and the concentration of supernatant was measured DNA concentration release by using a Nano-drop 2000c spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA.). Nucleic acid concentrations were calculated with the Nanodrop 2000 software (Zhou, 2016).

$$c = A*F \tag{3}$$

Where:

A: Absorbance in absorbance units A.

F: factor: F: factor in ng-cm/ml, F= 50 ng-cm/\(\mu\)l (dsDNA).

3.1.3.6 Model validation and experimental design

In order to predict the effect of cross-preventive factor of NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), and temperatures 65-85°C for 20 min on B. cereus spore targets, the experimental design was 3³ full factorial central composite experiment plan with 5 targets including spore germination rate, spore membrane permeability, spore membrane fluidity, DPA release, and DNA release. Statistical analysis of the model was performed to estimate the analysis of variance (ANOVA). In this study, the experimental plan consisted of 27 trials and the value of the dependent response was the mean of triplications. The significance (P<0.05) of the main effects (linear and quadratic), their interactions (linear, linear-quadratic, and quadratic-quadratic), regression coefficients and their determination coefficients were calculated and analyzed using the Design-Expert® soft-ware version 7.0.0 (Stat-Ease, Inc.2021, USA) statistic software.

The suitable condition from this part was confirmed to reveal the mechanism of *B. cereus* spore damage by Fourier-transform infrared spectroscopy (FTIR) analysis, Field Emission Scanning Electron Microscopy (FE-SEM) analysis, and genes expression. Furthermore, this condition was verified as a process critical control with sauerkraut fermentation and its pasteurization for monitoring the outgrowth of *B. cereus* spore and safety of aflatoxin B1 on 3.2 and 3.3.

3.1.3.7 Fourier-transform infrared spectroscopy (FTIR) analysis

Spectra were acquired with a Bruker FTIR Tensor 27 Hyperion equipment (Bruker, Karlsruhe, Germany). At a resolution of 4 cm-1 with 64 scans co-added at room temperature, all spectra of each sample were collected in the wave number range from 4000 to 400 cm-1 and 1900-800 cm-1, which can reflect information of all primarily functional groups of bacteria spore untreated and treated samples. Spectral acquisition data were performed using OPUS 7.0 software (Bruker, Karlsruhe, Germany) (Mahidsanan et al., 2017).

3.1.3.8 Field Emission Scanning Electron Microscopy (FE-SEM) analysis

To determine the efficacy of control measure on morphological of *B. cereus* ATCC 11778 spore, a FE-SEM study was performed using Zeiss AURIGA FE-SEM/FIB/EDX Germany, Furthermore, the morphological changes were observed by FE-SEM modified from the (Mustafa, 2010). The spores were collected by centrifugation (12000×g, 4 min at 4°C), then washed gently thrice with 10 mM/L PBS (pH 7.2), and fixed with 2.5% glutaraldehyde for 2 h at 4 °C. Then spores were washed with PBS twice. The dehydration process of spore was performed with 0, 30, 50, 70, 80, 90, 95 and 100% (v/v) of absolute ethanol in 10 min. The fixed spores were dried and gold-coated using Mini sputter coater (Model Leica EM ACE600, America) and then examined with a FE-SEM) (Mustafa, 2010, Kumari et al, 2016; Van Boeijen, 2010).

3.1.3.9 Heat and acid resistant genes expression

spore were determined by PCR analysis using specific primer on Table 3.1 Total genomic DNA from *B. cereus* spore on untreated and treated sample were extracted as described by CTAB method (Zhou et al., 1996). The sample were centrifuged at 5,000×g for 10 min to collect spores, and then resuspended in 525 μl of TE buffer content 10 mM Tris-HCl and 1 mM EDTA (pH 8). CTAB-chloroform extraction and isopropanol precipitation was used to extract DNA. First, 5 μl of proteinase K and 40 μl of lysozyme 50 mg/ml were added to the spore suspension then incubated for 1 h at 37°C, continuous added 30 μl of 10% sodium dodecyl sulfate and 100 μl of 10% *N*-lauroylsarcosine, then incubated for 30 min. The volume was adjusted to 3,000 μl by adding 1,420 μl of TE buffer and 420 μl of 5 M NaCl. 300 μl of preheated (65°C) 10% CTAB in 0.7 M NaCl was added to purify

DNA, and then was incubated for 10 min at 65°C and centrifuged at 10,000×g for 20 min. The DNA was resuspended in 100 ml of TE buffer for further step.

The 15 ng of extracted genomic DNA was amplified in a 30-μL reaction mixture containing 2X buffer (1X with 1.5 mM MaCl₂, 0.2 nM of each dNTP) by using the Kapa Taq DNA polymerase (KAPA Taq Ready mix PCR kit: USA), 10 pmol/μL of each PCR primer, PCR qualified water and 50 ng/μL of DNA. Thermal cycling was performed at an initial PCR denaturation at 95°C for 3 min, followed by 35 cycles of 95, 5 and 72°C for 30 sec, 30 sec and 2 min, respectively, and a final extension at 72°C for 5 min and hold 4°C. The primers were obtain from commercial source. The PCR products were separated by size using gel electrophoresis on 1.5% agarose gel, and then was run at 135 volts (where is subtract) for 40 min (Phamacia Biotech Model HE99x-15-1-5). A 1000 bp DNA ladder was used as a size standard in each run. Gel patterns were visualized by ethidium bromide staining and photographs taken by Gel Documentation (MacroVue UVis-20 Hoefer).

3.1.4 Assessment of cross-protective control measure condition for reducing AFB1 level

Aflatoxin B1 was obtained from Triology (Trilogy Analytical Laboratory Pvt. Ltd, USA). Stock solution of AFB1 $25\mu g/ml$ was prepared in acetonitrile/methanol and kept in the dark at $-20^{\circ}C$.

NB media was used as the model system for analysis of the of multiple factor of NaCl 2%, lactic acid 1.2%, temperature 82°C for 20 min NaCl on AFB1. The final concentration AFB1 on control and treatment sample were 200µg/kg. Aflatoxin B1 concentration was evaluated for the effecting on reduction level in NB media.

Table 3.1 Sequences of PCR primers targeting various virulent factor genes in this study.

Con torge	Function	Primer sequence (5'-3')	Length	Reference	Gene locus
Gentargi			bp		
DnaK	Heat and	F 5'-AGCCAACTCGTCGTGCATTA	1836	Designed	NC_004722
	acid	R 5'-ACATTGGACGCTCACCTTGT-3		for this	
	resistance			study	
BC1006	Acid	5'-GAAGCTCTTGA <mark>TA</mark> TGGGGGCA-3'	1143	Designed	NC_004722
	resistance	5'-GCGTAACGCTACACGCATAC-3'		for this	
				study	
hrcA	Heat	5'-GCTTACGGAACGTCAGCTCT-3'	1017	Designed	NC_004722
	resistance	5'-GTTCTTCCATCGGTACGCCA-3'		for this	
		4' 4 '4		study	
AbrB	Heat	5'-CGCCGTACTTTAGGTATTGCAG-3	285	Designed	NC_003997
	resistance	5'-AGATTTGCTCAGCGCCTTCT-3'		for this	
				study	

To determine the effect of multiple factor of NaCl, lactic acid and temperature on AFB1 on laboratory scale, each 200µg/kg Aflatoxin B1 was inoculated in NB containing: NaCl 2% (1); lactic acid 1.2% (2); temperature 82°C for 20 min (3); combination of NaCl 2% and lactic acid 1.2% (4); multiple combination as control measure condition NaCl 2%, lactic acid 1.2 %, and temperature 82°C for 20 min (5); and without treatment as control sample (6).

3.2 Verification of cross-preventive condition for monitoring the outgrowth of *B. cereus* spore and safety of aflatoxin B1

3.2.1 Sauerkraut model preparation and sampling

Fresh white cabbages (*Brassica oleracea* L. var. *capitata* cv. Bronco) used in this study were obtained from a local supermarket in Nakhon Ratchasima province (Thailand) and stored at 4°C until further use.

The cabbages were trimmed of outer leaves, washed, drained and cut into strips (2-3 cm x 6-8 cm). Brine solution of 2% of NaCl was added in to 1 kg shredded white cabbages, mixed, packed into jars (1.5 L) and pressed tightly together in the fermentation vessels and covered with a food-grade plastic film on which water was poured to prevent air from entering, and kept at ambient temperature 25°C running 7 days of sauerkraut fermentation.

During the fermentation, 10g sample (liquid and solid parts) of sauerkraut was withdrawn from 0, 6, 12, 18, 24 hours then every 24 hours for 7 days of fermentation period. Then the samples were homogenized and filted. The filtrate were used for measurements of pH value, titratable acidity, lactic acid, acetic acid, and analysis of microbiological changes including LAB, total viable microorganism, molds and yeasts, and *B. cereus*.

3.2.2 Challenging test of *B. cereus* spores

Cabbage fermentation was carried out using two methods, namely, spontaneous fermentation and inoculation with either *B. cereus* or *B. cereus* combined with lactic acid 0.2% (w/v). For fermentation challenged test, 5.1 log CFU/g *B. cereus* spore (1) and 5.1 log CFU/g *B. cereus* spore combined with lactic acid 0.2% (w/v) (2) were inoculated in to cabbage. The cabbage fermentation and sampling was described as 3.2.1.

3.2.3 Challenging test of Aflatoxin B1

The sauerkraut fermented in this study include (1) spontaneous fermentation, (2) inoculated with 200 μ g/kg Aflatoxin B1, and (3) inoculated 200 μ g/kg Aflatoxin B1 combined with lactic acid 0.2% (w/v). The cabbage fermentation and sampling was described as 3.2.1.

3.2.4 Microbiological analysis

The sample was blended in 90 ml sterile NaCl solution (0.85% (w/v)) in a 500 ml Erlenmeyer, and then serial dilution were made. The lactic acid bacteria were enumerated by anaerobically culturing on MRS agar for 2-3 days at 25-30°C. Total viable count was counted on plate count agar which were incubated at 37°C for 2 days. *B. cereus* were grown on phenol red egg yolk polymyxin agar base, grown for 1 days at 37°C. Molds and yeasts were grown on Potato Dextrose Agar on 7 days at 25°C. All microbiological analyses were made in triplicate.

3.2.5 Physico-chemical measurement

3.2.5.1 **pH** value

The decrease in pH of the sample was determined using a pH meter (pH Lab 865, Xylem Analytics Germany Sales GmbH & Co. KG).

3.2.5.2 Titratable acidity

Titratable acidity of fermented white cabbages fermentation expressed as percent of lactic acid was determined by titrating the brine with 0.1N NaOH with phenolphthalein (0.1% w/v in 95% ethanol) as the indicator (AOAC, 1990). The concentration of titratable acidity (%) was calculated by using following formula (4).

Titratable acidity (%,
$$w/v$$
) = $\frac{ml \ of \ 0.1N \ NaOHx0.009}{Sample \ volum \ (ml)}$ (4)

Titratable acidity is expressed as % lactic acid (CH₃CH₂COOH, MW = 90).

3.2.5.3 Lactic acid and acetic acid measurement by high performance liquid chromatography (HPLC)

Lactic acid and organic acid in the sample were analyzed by high performance liquid chromatography (HPLC, Agilent, USA). The samples were centrifuged at 12000×g for 10 min at 4°C, then the supernatant was filtered through a 0.2μm filters membrane. A 20-μl sample of which was injected into an anion exchange Aminex HPX-87H column (300x7.8 mm; Bio-Rad, Hercules, CA, USA), which connect to a refractive index detector UV detector for organic acids at 205 nm (Agilent, USA). Elution was performed at 45°C with 5 mM H₂SO₄ using as a mobile phase at a flow rate of 0.5 ml min⁻¹ for separate lactic acid and acetic acid. The quantification analysis of lactic acid and acetic acid was performed by comparison of their retention times and peak areas with external standards which were prepared from various concentrations of the respective reference substances analyzed under the same conditions (Papadelli et al., 2015; Xiong et al., 2014).

3.2.6 Quantification of AFB1 reduction by Enzyme-Linked Immunosorbent Assay (ELISA) method

Ten gram of fermented cabbage samples (solid and liquid part) was extracted by using 90 ml of 70% methanol (v/v). The samples were thoroughly mixed for 3 min. Aliquots of the solvent extracts were decanted and filtered using Whatman#1 filter paper. The samples were analyzed using a commercial ScreenEZ [®] Aflatoxin ELISA Test Kit procedure, which was described by the producer company (SIAM Inter Quality Co., Ltd. Bangkok, Thailand). Microtitre plate Enzyme-Linked Immunosorbent Assay (ELISA) reader (MultiskanTM GO Microplate Spectrophotometer, United States) and AFB1 test kit

were used to determine. The absorbance at 450 nm was recorded (Fan, 2018).

Sample preparation was carried out according to the instructions from the manufacturer.

3.3 Verification of pasteurization process control of *B. cereus* spore and aflatoxin B1 hazard safety

3.3.1 Pasteurization on sauerkraut models

The spontaneous fermentation and all challenged test at the end of 7 days of fermentation time were pasteurized by using a water bath Shel lab WSBR27 (Lab Leader Co. Ltd, NY 14519 USA) at 82°C on 20 min. Then immediately cooled in ice-water to stop reaction, sauerkraut was verified in term of microbiological and physico-chemical parameter of brine sauerkraut.

3.3.2 Safety parameter

3.3.2.1 Lactic acid bacteria

The method has been described on 3.2.4.

3.3.2.2 Physico-chemical measurement

The method has been descried on 3.2.5.

3.4 Statistic analysis

All experiments and analyses were conducted in triplicate. Data analysis was carried out by ANOVA (SPSS software, version 16). All results were expressed as mean values \pm SD. Treatment means were separated by Duncan's Multiple Range Test (DMRT). P values < 0.05 were considered to be significant.

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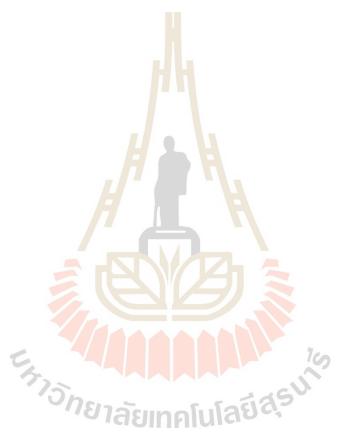
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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Assessment of cross-preventive condition for sub-lethal stress of

B. cereus spore targets

To assess the cross-preventive factors to sub-lethal *B. cereus* spore under NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), pasteurization temperatures 65-85°C for 20 min, the predictive microbiological RSM model would be designed for the control measure point achieving the stress of *B. cereus* spore targets. The combination criteria such as spore germination rate, spore membrane permeability, spore membrane fluidity, DPA release, DNA release, and resistant genes (*Dna*K, *hrc*A, *Abr*B and BC1006) were evaluated which the hazard *B. cereus* spore viability would be considered under microbiological standard requirement of fermented vegetable product safety. Thus, the result of those predictive model were validated as show below.

4.1.1 Predictive microbiological modeling of *B. cereus* spore under multiple stress conditions

In order to validate the efficiency of combination preventive factor control, the combination criteria including spore germination rate, spore membrane permeability, spore membrane fluidity, DPA release, DNA release were considered as 4.1-4.5.

4.1.1.1 Spore germination rate

Vegetative cells are formed by spore germination followed by

a more extended outgrowth phase. The efficiency of cross-preventive conditions on *B*. *cereus* spore was assessed above.

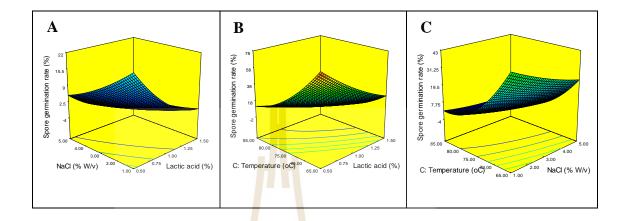


Figure 4.1 Response surface showing relative effect on spore germination rate (%) of *B. cereus* ATCC 11778 at different combinations of temperatures (65°C, 75°C, 85°C for 20 min), NaCl (1%, 3%, 5% (w/v)) and lactic acid (0.5, 1.0, 1.5% (w/v)). (A) Effect of NaCl versus lactic acid at constant temperature of 82°C for 20 min; (B) Effect of lactic acid versus temperature at constant NaCl 2%; (C) Effect of NaCl versus temperature at constant lactic acid 1.2%.

As Figure 4.1, the spore germination rate decreased with an increase in temperatures, NaCl and lactic acid concentration (P<0.05). It could be caused by interference with the cell wall, cell membrane, metabolic enzymes, protein synthesis system, under cross-preventive stresses (Setlow, 2014; Korza et al., 2016; Trunet et al., 2017). At the low pH, weak acid preservatives may affect the microbial viability, ATP levels to maintain pH homeostasis (Mira et al., 2010). Inside the cell and weak acids environmental factor—generally encounter a higher pH due to the cell buffers,

dissociate and become toxic, which ultimately inactivated the spore due to the acidification stress responses of the cell interior (Leguerinel and Mafart, 2001; Beales, 2004; Pandey et al., 2016).

However, no significant differences were observed in germination percentages among samples studied (P \geqslant 0.05).

4.1.1.2 Spore membrane permeability

Hydrophobic crystal violet is general penetrate weakly to the outer membrane but it has been found to penetrate impaired cell membrane, which can detect the microbial membrane permeability (Devi et al. 2010). To determine the potential mechanisms of cross-preventive factors control on spore forming bacteria, their influence on membrane permeability was determined using the crystal violet assay. The percentages of crystal violet uptake by whole spores could reflect a difference in the amount of dye bound in permeability of the spore envelope Figure 4.2 shows that the spore up take (%) of crystal violet increased with an increase of temperature, NaCl concentration while pH value decreased (p<0.05). It suggested that alteration of cross-preventive factors of temperature, lactic acid and NaCl may correlate to membrane permeability, and led to B. cereus death. However, the present findings can not only be one parameter for studying membrane active molecules.

4.1.1.3 Spore membrane fluidity

Figure 4.3 shows the fluorescence intensity of *B. cereus* ATCC 11778 spore after different treatments. The fluorescence intensity increased with an increase in temperature, NaCl concentration and a decrease of pH value (P<0.05). The DPH fluorescent probe can embed into the phospholipid bilayers and is sensitive to alterations in microbial membrane fluidity. The fluidity of the spore membrane

correlated to the probe fluorescence intensity; as the membrane fluidity decreased, the intensity increased (Stubbs and Williams, 1992; Denich, Beaudette, Lee, and Trevors, 2003; Munishkina and Fink, 2007; Hwang et al., 2011). The DPH treated spore increased the membrane fluidity when compared to the untreated spore (P<0.05). These results are correlated to the spore germination rate (4.1.1.1).

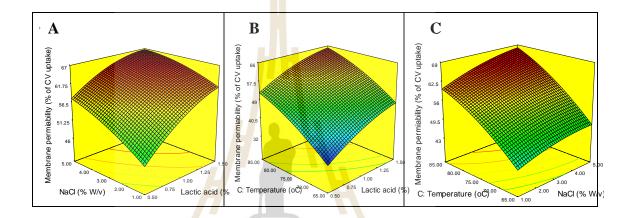


Figure 4.2 Response surface showing relative effect on spore membrane permeability of *B. cereus* ATCC 11778 spore at different combinations of temperatures (65°C, 75°C, 85°C for 20 min), NaCl (1%, 3%, 5% (w/v)), and lactic acid (0.5, 1.0, 1.5% (w/v)). (A) Effect of NaCl versus lactic acid at constant temperature of 82°C for 20 min; (B) Effect of lactic acid versus temperature at constant NaCl 2%; (C) Effect of NaCl versus temperature at constant lactic acid 1.2%.

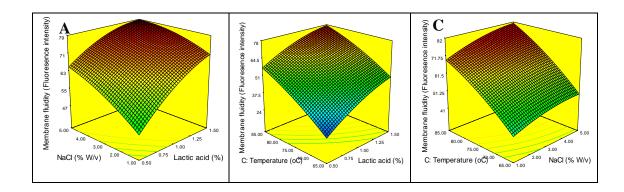


Figure 4.3 Response surface showing relative effect on spore membrane fluidity of *B. cereus* ATCC 11778 spore at different combinations of temperatures (65°C, 75°C, 85°C for 20 min), NaCl (1%, 3%, 5% (w/v)) and lactic acid (0.5, 1.0, 1.5% (w/v)). (A) Effect of NaCl versus lactic acid at constant temperature of 82°C for 20 min; (B) Effect of lactic acid versus temperature at constant NaCl 2%; (C) Effect of NaCl versus temperature at constant lactic acid 1.2%.

4.1.1.4 Measurement of dipicolinic acid (DPA) release

The release of DPA cooperated with the activation of the spore cortex by lytic enzymes, which are responsible for degradation of the cortex and changes in inner membrane organization, alterations in the structure of channel proteins, or associated DPA binding proteins (Reineke et al, 2013; Wang, 2017). The Figure 4.4 shows the effects of multiple condition on the release of DPA in *B. cereus* spore. The effectiveness of NaCl 2% (w/v) affected the DPA release increased with a lower pH towards the acidity side when increased the concentration of lactic acid and increased in temperature. Multiple stress of NaCl, lactic acid, and mild temperatures acted on the spore's inner membrane to cause DPA release, targeting either membrane protein.

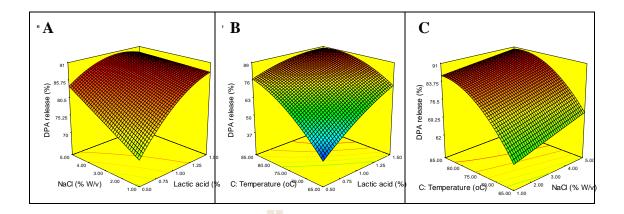


Figure 4.4 Response surface showing relative effect on DPA release of *B. cereus* ATCC 11778 spore at different combinations of temperatures (65°C, 75°C, 85°C for 20 min), NaCl (1%, 3%, 5% (w/v)) and lactic acid (0.5, 1.0, 1.5% (w/v)). (A) Effect of NaCl versus lactic acid at constant temperature of 82°C for 20 min; (B) Effect of lactic acid versus temperature at constant NaCl 2%; (C) Effect of NaCl versus temperature at constant lactic acid 1.2%.

4.1.1.5 Measurement of DNA release

Ozone is thought to kill bacteria bymembrane damage followed by cell lysis. The release of genomic DNA is an important indicator of bacterial lysis. Therefore, to further detect the integrity of spore membranes after cross-preventive stress. Figure 4.5 shows the concentration of DNA release by *B. cereus* ATCC 11778 spore after different treatments. The DNA concentration release increased with an increase of temperature, NaCl and lactic acid concentration and a decrease of pH value (P<0.05). Spore were damaged by combination factors to render the spores defective in germination, perhaps because of damage to the spore's inner membrane. We believe

that bacterial lysis was the consequence of stress response, which eventually releases the DNA into the environment.

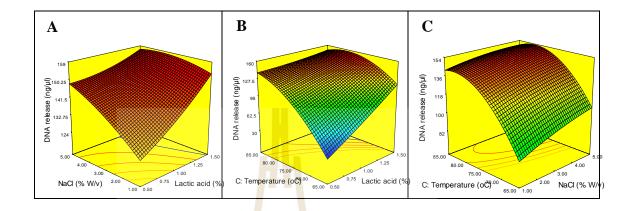


Figure 4.5 Response surface showing relative effect on DNA release of *B. cereus*ATCC 11778 spore at different combinations of temperatures (65°C, 75°C, 85°C for 20 min), NaCl (1%, 3%, 5% (w/v) and lactic acid (0.5, 1.0, 1.5% (w/v)). (A) Effect of NaCl versus lactic acid at constant temperature of 82°C for 20 min; (B) Effect of lactic acid versus temperature at constant NaCl 2%; (C) Effect of NaCl versus temperature at constant lactic acid 1.2%.

There is significant interest in the use of hurdle cross preventive factors to eliminate or reduce levels of bacterial. Response Surface Methodology (RSM) was used to predict the combination preventive factors for controlling *B. cereus* ATCC 11778 spore survival after pasteurization (temperatures, 65-85°C for 20 min) under the condition of lactic acid (0.5-1.5% (w/v)) and NaCl (1-5% (w/v)) on multiple sub-lethal of spore targets. The control measurement was selected, based on the combination criteria at each factor range using the statistical software

(Design-Expert® soft-ware version 7.0.0, USA). Our results indicated that NaCl 2% (w/v), lactic acid 1.2% (w/v), pasteurization temperature 82°C for 20 min could control the outgrowth of *B. cereus* spore. Thus, those hurdle effects of cross-preventive factor of NaCl, lactic acid and pasteurization temperature on spore targets could be satisfactorily microbial predicted under the current experiment condition, and those microbiological models examined could serve as a tool for this purpose.

4.1.2 Mechanism of *B. cereus* spore damage under cross-preventive control measure condition

Spore killing by ozone seems to render the spores defective in germination, perhaps because of damage to the spore's inner membrane.

The mechanism of cross-preventive condition was also assessed under FTIR spectra (Figure 4.6) and FE-SEM chromatograph (Figure 4.7), and heat and acid resistant gene expression (Figure 4.8-4.12).

4.1.2.1 FTIR spectra

The FT-IR was used to determine the mode of action of *B. cereus* spores under their cross-preventive factors for further scrutinize their spectral identities. The Figure 4.6F shows that the wavelength number of 1075, 1232, 1383, 1454, 1633 were shifted when compared to those of fresh spore (A), corresponding to the *B. cereus* spore surface composition of fatty acid, protein mixed (α-helix and/or β-sheet of protein with phosphodiesters), carbohydrates and/or polysaccharide (peptidoglycan backbones), respectively. For the absorbance band of treated spore (Figure 4.6F), the intensities (1633-1000 cm⁻¹) decreased compared to those of the untreated spore to which the spectrum contributes protein denaturation and

carbohydrate deformation after cross-preventive treatment (Ghosh et al., 2015; Li et al., 2011; Santivarangkna et al., 2007). Spore compositions were damaged by cross-preventive condition NaCl 2% (w/v), lactic acid 1.2% (w/v), and pasteurization at 82°C for 20 min.

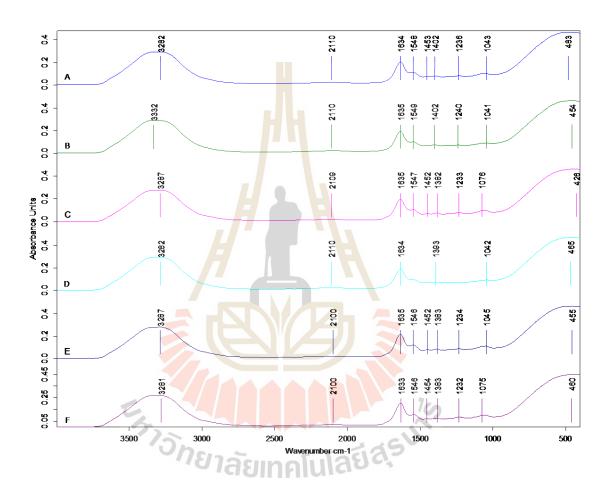


Figure 4.6. FTIR spectra of *B. cereus* ATCC 11788 spores untreated (A) and treated by NaCl 2% (B), lactic acid 1.2% (C), temperature 82°C for 20 min (D), lactic acid 1.2% + NaCl 2% (E) and combination NaCl 2% + lactic acid 1.2% + temperature 82°C for 20 min (F), respectively.

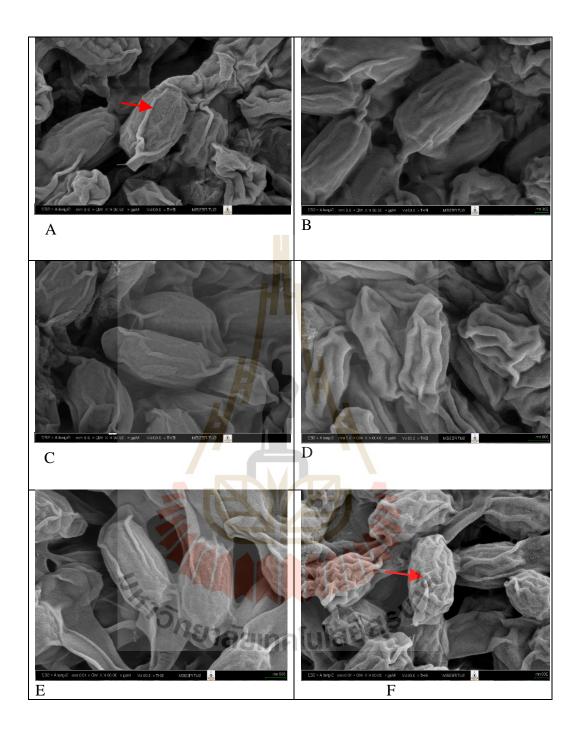


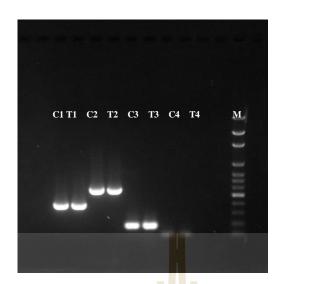
Figure 4.7 FE-SEM image of *B. cereus* ATCC 17788 spores untreated (A) and treated by NaCl 2% (B), lactic acid 1.2% (C), temperature 82°C for 20 min (D), lactic acid 1.2%+NaCl 2% (E) and combination NaCl 2%+lactic acid 1.2% + temperature 82°C for 20 min (F) respectively. The scale bars in the panels are 300 nm.

4.1.2.2 FE-SEM

As previous mentioned, the cross-preventive stress of *B. cereus* evaluated from these predictive microbiological model were proved under FE-SEM to confirm the damage of spore characteristics. The Figure 4.7F reveals that the combination factors of 2% (w/v) NaCl and 1.2% (w/v) lactic acid and pasteurization at 82°C for 20 min affected the morphological appearances spore and caused the irregular sharp, when compared to those of fresh spores (Figure 4.7A).

4.1.2.3 Heat and acid resistant genes expression

Due to consider the heat and acid resistant genes expression (Figure 4.8-4.12), the heat resistance genes *DnaK*, *hrcA*, *AbrB* were more effected under control measure condition (NaCl 2%, lactic acid 1.2% and temperature 82°C for 20min) than individual effect. However, the acid resistant gene of BC1006 was not expressed because of 1.2% w/v of lactic acid, as same as the combination cross-preventive stress of 2% (w/v) of NaCl, 1.2% (w/v) lactic acid had effect the BC1006 expression which similar to three cross-preventive factor condition of NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), pasteurization temperatures 65-85°C for 20 min. This phenomenon confirmed heat, acid, and salt stress can induce the outgrowth of B. cereus spore. Those cross-preventive condition had changed the stress gene targets, cell wall, cell membrane its structure could be destroyed by lactic acid, heat which protein could be accumulated due to unfold and denaturation (Papadimitriou et al., 2016). In addition, the enzyme and metabolic activity were inactivated and the genes were also not expressed. Therefore, the control measure point of NaCl 2% (w/v), lactic acid 1.2% (w/v), pasteurization temperature 82°C for 20 min condition could control the growth B. cereus and had gained to prototype of cross-preventive condition for using in standard safety operating process in sauerkraut fermentation.



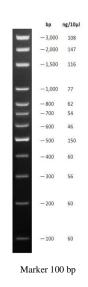


Figure 4.8 PCR assay products were separated by agarose gel electrophoresis. Lane C1, C2, C3, C4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 control genes respectively; Lane T1, T2, T3, T4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 treatment at control measure condition NaCl 2%. M molecular size standards.



Figure 4.9 PCR assay products were separated by agarose gel electrophoresis. Lane C1, C2, C3, C4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 control genes

respectively; Lane T1, T2, T3, T4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 treatment at lactic acid 1.2%. M molecular size standards.

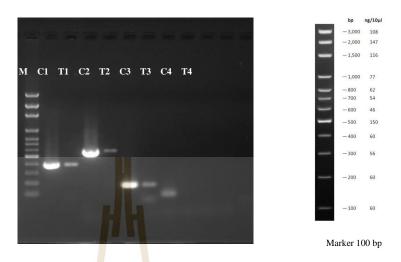


Figure 4.10 PCR assay products were separated by agarose gel electrophoresis. Lane C1, C2, C3, C4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 control genes respectively; Lane T1, T2, T3, T4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 treatment at combination condition (NaCl 2%, lactic acid 1.2%). M molecular size standards.

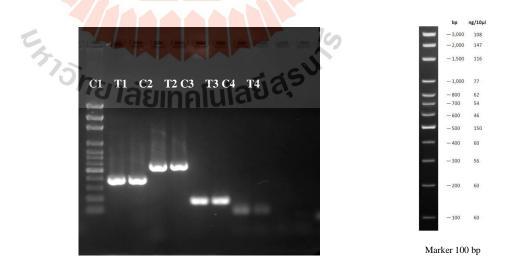


Figure 4.11 PCR assay products were separated by agarose gel electrophoresis. Lane C1, C2, C3, C4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 control genes

respectively; Lane T1, T2, T3, T4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 treatment at temperature 82°C for 20 min. M molecular size standards.



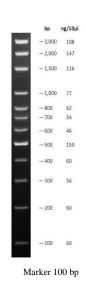


Figure 4.12 PCR assay products were separated by agarose gel electrophoresis. Lane C1, C2, C3, C4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 control genes respectively; Lane T1, T2, T3, T4 ran as *Dna*K, *hrc*A, *Abr*B and BC1006 treatment at control measure condition (NaCl 2%, lactic acid 1.2%, temperature 82°C for 20 min). M molecular size standards.

4.2 Verification of cross-preventive condition for monitoring the outgrowth of *B. cereus* spore

The sauerkraut model challenged with surrogate high risk *B. cereus* spore and aflatoxin B1 chemical hazard were verified under the control measure condition.

4.2.1 B. cereus, lactic acid bacteria, total viable count, molds and yeasts profile

The population dynamics of the different microbial groups in the brines during the processes are presented in Figure 4.13-4.16 as viability of *B. cereus*, lactic

acid bacteria, total viable count, molds and yeasts count, respectively.

In all cases, lactic acid bacteria reached their maximum within 2-3 days of fermentation (from 3.5-3.6 log CFU/g to 8.0-8.1 log CFU/g) then decreased to approximately 7.4-7.5 log CFU/g until the end of fermentation as Figure 4.14. In term of total viable count, populations increased steadily from the onset of fermentation, reaching a maximum value from 4.2-4.4 log CFU/g to 8.1-8.2 log CFU/g after 4 days of fermentation and then decreased to approximately 7.7-7.9 log CFU/g until the end of fermentation (Figure 4.15). Molds and yeasts showed load approximately 3.6-3.7 log CFU/g at the 0 hours to 8.1-8.3 log at the 7 days of fermentation (Figure 4.16). In case of B. cereus (Figure 4.13) it was not observed under spontaneous sauerkraut fermentation. The sample of cabbage which inoculated 5.1 log CFU/g of B. cereus spore, initially, a slightly increased to 5.2 log CFU/g after 2 days. On the other hand, it is similar trend to be evident in the control process of the sauerkraut fermentation inoculated with 5.1 log CFU/g of B. cereus spore and 0.2% (w/v) of lactic acid, number of B. cereus spores were reduced to 5.0 log CFU/g. In all challenge process groups, the B. cereus survivor was approximately 4.4-4.9 log CFU/g. In some outbreaks lower numbers in foods have been reported (3.0 to 4.0 log CFU/g) (EFSA, 2015). Many researches indicated that B. cereus can survive in several fermented products such as sour maize beverage (Byaruhanga et al., 1999), fermented soy bean (Nout et al., 1999), tempeh (Nout et al., 1987), fermented sauce-based salads (Bonestroo et al., 1993), fish sausage (Aryanta et al., 1991), fermented olive (Panagou, 2008) and fermented rice (Yang et al., 2008).

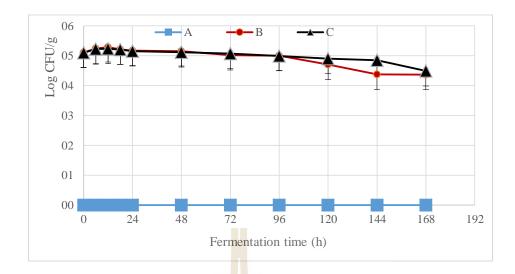


Figure 4.13 Growth rate of *B. cereus* during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

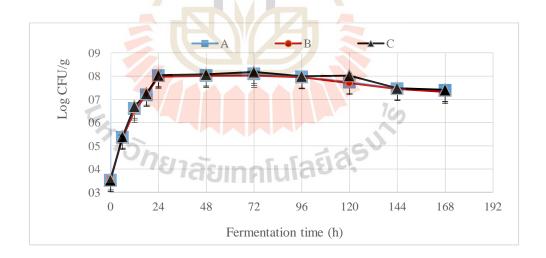


Figure 4.14 Growth rate of Lactic acid bacteria during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

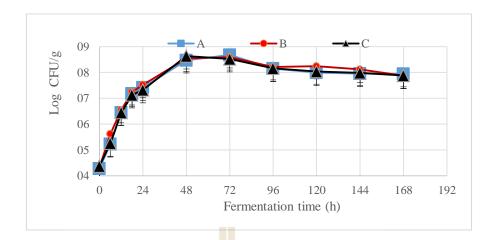


Figure 4.15 Growth rate of Total viable count during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

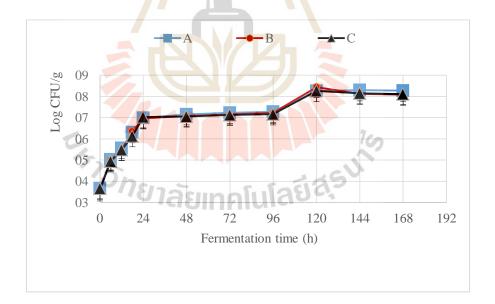


Figure 4.16 Growth rate of molds and yeasts during brine fermented cabbage at 25°C:
(A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore,
(C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%.
Error bars indicate standard deviations, based on triplicate.

4.2.2 Physico-chemical measurement

The survival of *B. cereus* during sauerkraut fermentation is reported here for the knowledge basis of the pathogen growth. The inactivation of *B. cereus* could be attributed to the presence of organic acids such as lactic acid, acetic acid (Figure 4.9), acid acetic (Figure 4.10). Salt concentration could be another inhibiting factor, although B. cereus has been reported to resistant up to 7.5% (w/v) NaCl (Johnson, 1984). Lactic and acetic acids affect the growth and survival of B. cereus which genes can induce acid resistance and/or acid tolerance (Foster, 2000). The extrinsic factors (temperature, gas atmosphere), intrinsic factors (organic acids, nutrients, food matrix, redox potential, acidity, and antimicrobial substances O2, water activity), and implicit factors (microbial growth rates and synergistic or antagonistic interactions among microorganisms) affect the B. cereus spore. During sauerkraut fermentation, the pH of brine fell within the range of 3.4 to 3.65 which considered to cause acid habituation of food-borne pathogens. The residual population of B. cereus 4.4-4.9 log CFU/g also could be caused the small changes in pH and titratable acidity during all processes (Figure 4.17-4.18). These changes could be attributed to the buffering capacity of the brine and the limited amount of fermentation.

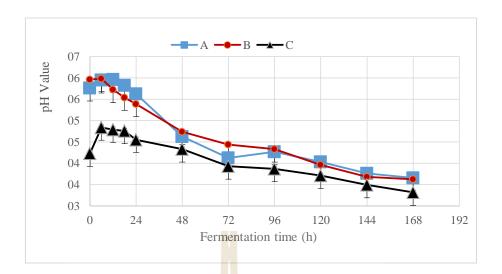


Figure 4.17 Change of pH during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

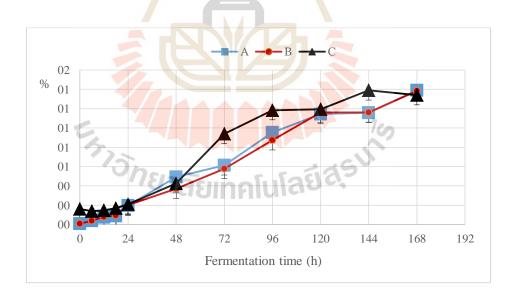


Figure 4.18 Change of titratable acidity (as % lactic acid) during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermetation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

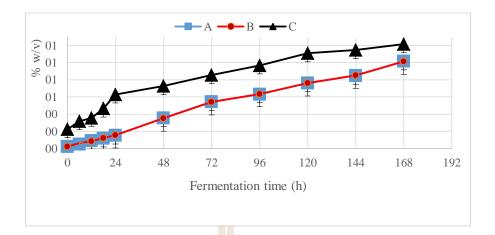


Figure 4.19 Change of lactic acid during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

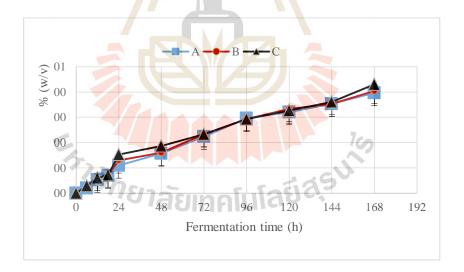


Figure 4.20 Change of acetic acid during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B. cereus* spore, (C) inoculated fermentation with *B. cereus* spore and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

4.3 Verification of cross-preventive condition for monitoring safety of aflatoxin B1

4.3.1 Effect of NaCl, lactic acid and temperature on Aflatoxin B1 concentration reduction

Aflatoxin B1 (AFB1) is mycotoxin and contaminates poorly to store food products. This study was carried out to investigate the potential activity of lactic acid fermentation in reducing AFB1 level in laboratory and fermented sauerkraut models under cross-preventive factors as proved previously in 3.1. Control measure condition was NaCl 2%, lactic acid 1.2% and temperature 82°C for 20min, which these factors could control the high risk hazard of spore forming *B. cereus* surrogate pathogen. The reults shows that the control measure condition (NaCl 2%, lactic acid 1.2% and temperature 82°C for 20min) approximately removed 51.209% (102.471 μg/kg) of aflatoxin B1, whereas individual effect of NaCl 2%, lactic acid 1.2%, temperature 82°C for 20 min and combination NaCl 2% with lactic acid 1.2% were 0.257% (0.515μg/kg), 27.460 (54.920μg/kg), 1.560% (3.119 μg/kg), 37.084% (74.168 μg/kg) of AFB1 reduction respectively. It indicates that multiple preventive factor increased in percentage of AFB1 reduction (P<0.05).

4.3.2 Verification of cross-preventive condition for monitoring safety of aflatoxin B1

Cabbage was challenged with final concentration of 200 μ g/kg AFB1, and fermented, with and without adding lactic acid 0.2% for 7 days at 25°C. The concentration of AFB1, lactic acid bacteria, lactic acid, acetic acid, pH and titratable acidity of cabbage fermentation were monitored from 0. 6, 12, 18, 24 hours, and then every 24 hours for 7 days of fermentation periods. During cabbage fermentation was

critical to determine the LAB for detoxification AFB1 which the percentage of AFB1 production was increased (Figure 4.27).

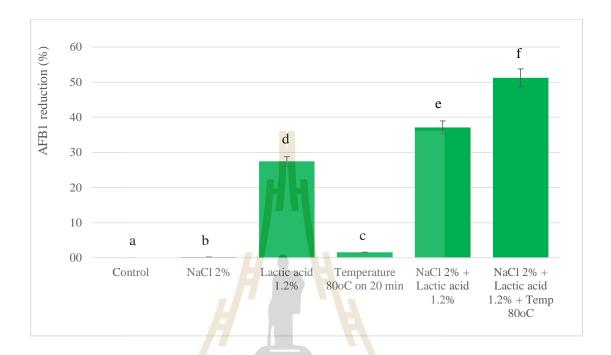


Figure 4.21 Percentage of AFB1 reducing at different treatments. The bars refer to the mean value (n=3) and standard deviation. The different letter, a, b, c, ..., and f between groups indicate significant differences at p<0.05.

A significant decrease (p<0.05) in the level of AFB1 was observed from 67.286% (134.573 μg/kg) in the days 7. In contrast, the combined treatment with lactic acid 0.2% after 7 days of fermentation was 73.372% (146.745 μg/kg). The LAB. Lactic acid, acetic acid and titratable acidity were observed. Thus, pH drop is a key indicator of bacterial activity in fermented foods (Xiong et al., 2015). This corresponded to the reduction of AFB1 level (Figure 4.23-4.27) within the same time period owing to mycotoxin transformation AFB1 (Galvano et al., 2001; El Nezami et al., 2002; Nyamete et al., 2016). In recent years, strategies involving use of microorganisms for this purpose

have been investigated. The mechanism of decontamination by LAB is reportedly via binding of mycotoxins to the cell wall of bacteria and/or conversion of mycotoxins into less or non-toxic forms (Gourama and Bullerman, 1995; Thyagaraja and Hosono, 1994). The cell wall of one of the most studied LAB, *Lb. rhamnosus*, has polysaccharides where it binds aflatoxins. According to Haskard et al. (2001), reported that bacteria cell wall has a significant impact to bond formation. AFB1 is bound to bacteria cells by weak non-covalent interactions, such as hydrophobic AFB1 pockets on the bacterial surface. In addition, Magnusson et al. (2003) indicated that three mechanisms can explain the antimicrobial effectiveness because of organic acid (lactic acid, acetic acid) and antagonist production.

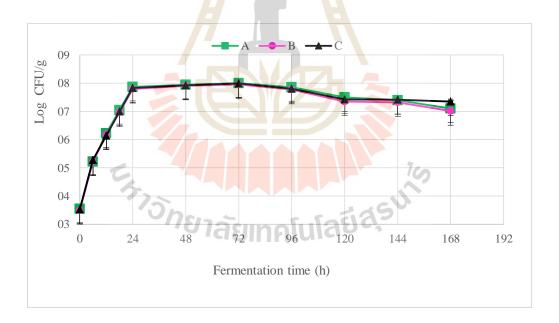


Figure 4.22 Growth rate of lactic acid bacteria during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with *B*. aflatoxin B1, (C) inoculated fermentation with aflatoxin B1 and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

More investigation is still needed for the interactions between cross-preventive factor to provide insight on fermentation conditions which can be optimized for achieving greater mycotoxins reduction.

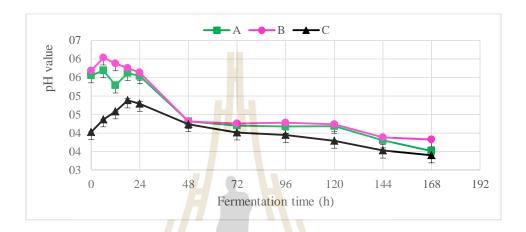


Figure 4.23 Change of pH during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with aflatoxin B1, (C) inoculated fermentation with aflatoxin B1 and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

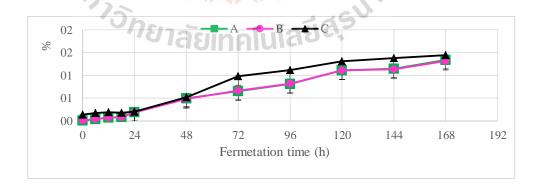


Figure 4.24 Change of titratable acidity (as % lactic acid) during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation

with aflatoxin B1, (C) inoculated fermentation with aflatoxin B1 and lactic acid 0.2%. Error bars indicate standard deviations, based on on triplicate.

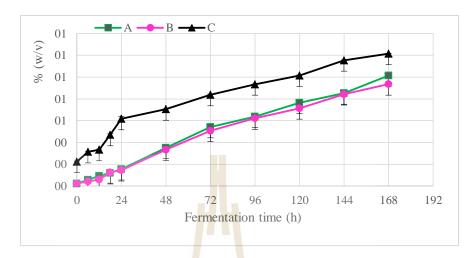


Figure 4.25 Change of lactic acid during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with aflatoxin B1, (C) inoculated fermentation with aflatoxin B1 and lactic acid 0.2%. Error bars indicate standard deviations, based on on triplicate.

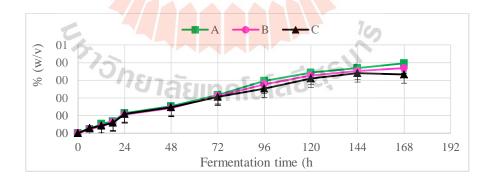


Figure 4.26 Change of acetic acid during brine fermented cabbage at 25°C: (A) spontaneous process, (B) inoculated fermentation with aflatoxin B1, (C) inoculated fermentation with aflatoxin B1 and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate.

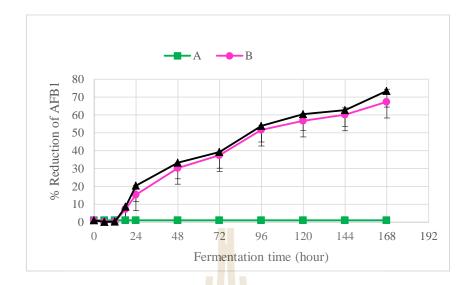


Figure 4.27 Percentage of aflatoxin B1 reduction during brine fermented cabbage at 25°C within 168 hours. (A) Spontaneous process, (B) inoculated fermentation with aflatoxin B1, (C) inoculated fermentation with aflatoxin B1 and lactic acid 0.2%. Error bars indicate standard deviations, based on triplicate. Note: spontaneous process, AFB1 was N/A.

4.4 Verification of pasteurization process control of *B. cereus* spore and aflatoxin B1 hazard safety

At present, quantitative of corrective and/or preventive action, according to quality assurance systems such as FSMA, QMRA, ISO 31000 and HACCP is a significant approach of which relevant to present microbial hazard and chemical in safety risk management. In order to minimize the undesirable amount of over cooking, lethal *B. cereus* spore, toxin reducing level, the hurdle technology is still remained.

4.4 Verification of pasteurization process control of *B. cereus* spore and aflatoxin B1 hazard safety

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Table 4.1 Physico-chemical and microbiological changes at the end of fermentation 7 days and after pasteurization at 82°C on 20 min.

		LAB	TVC	M & Y	B.cereus	LA	\mathbf{AC}	pН	TA
Spontaneous	End of	7.4 ±	8.0 ±	8.3 ±	ND	1.016 ±	0.397 ±	3.657 ±	1.277 ±
fermentation	fermentation	0.021	0.061	0.040		0.053	0.058	0.231	0.066
	7 days								
-	After	ND	ND	ND	ND	1.133 ±	0.410 ±	3.833 ±	1.250 ±
	pasteurization					0.042	0.010	0.038	0.000
Inoculated	End of	7.3 ±	7.3 ±	8.1 ±	4.367 ±	1.012 ±	0.430 ±	3.623 ±	1.383 ±
fermentation with	fermentation	0.082	0.082	0.040		0.020	0.010	0.153	0.012
B. cereus spore	7 days				0.045				
-	After	ND	ND	ND	2.02 ±	1.140 ±	0.397 ±	3.757 ±	1.423 ±
	pasteurization				0.012	0.025	0.058	0.006	0.058
Inoculated	End of	7.5 ±	7.9 ±	8.1 ±	4.490 ±	1.214 ±	$0.407 \pm$	3.317 ±	1.340 ±
fermentation	fermentation	0.015	0.125	0.038	0.000	0.005	0.058	0.006	0.182
with B. cereus	7 days								
spore and lactic	After	ND	ND	ND	ND	1.238 ±	0.420 ±	3.377 ±	1.323 ±
acid 0.2%	pasteurization					0.002	0.010	0.116	0.0056

LAB: Lactic acid bacteria (Log CFU/g), TVC: Total viable count (Log CFU/g),

M&Y: Molds and yeasts (Log CFU/g), B. cereus: Bacillus cereus (Log CFU/g)

TA: Titratable acidity (% (w/v)), LA: Lactic acid (% w/v), AC: Acetic acid (% (w/v)).

4.4.2 Verification of pasteurization process control of Aflatoxin B1

Pasteurization of natural lactic acid fermentation of cabbage may potentially decreased the natural toxins occurring in food vegetable fermentation and also improved food safety. From the Table 4.2, the combined treatment with lactic acid 0.2% after 7 days of fermentation, the level of AFB1 reduction reached 84.07% ($168.14~\mu g/kg$).

Table 4.2 Physico-chemical and microbiological changes at the end of fermentation 7 days and after pasteurization at 82°C on 20 min.

		A	2	A				oxin B1 ucing
Fermen	LAB	LA	LA AC		TA (%)	Percentage %	Concentra tion µg/kg	
Spontaneous	End of	7.1 ±	1.015 ±	0.397 ±	3.513 ±	1.340 ±	NA	NA
fermentation	fermentation	0.095	0.005	0.006	0.006	0.026		
	7 days							
	After	ND	1.013 ±	0.429 ±	4.166 ±	1.377 ±	NA	NA
	pasteurization		0.005	0.009	0.0068	0.008		
Inoculated	End of	7.0 ±	0.935 ±	0.370 ±	3.827 ±	1.317 ±	67.287 ±	134.573 ±
fermentation	fermentation	0.026	0.005	0.010 ^l	0.060	0.006	0.427	0.855
with aflatoxin	7 days	-610	IIIIII	AIG				
B1								
	After	ND	0.985 ±	0.394 ±	3.726 ±	1.309 ±	75.6371	151.274
	Pasteurization		0.009	0.003	0.0021	0.002	± 0.641	±1.282
Inoculated	End of	7.4 ±	1.289 ±	0.334 ±	3.408 ±	1.415 ±	73.372 ±	146.745 ±
fermentation	Fermentation	0.028	0.002	0.034	0.008	0.007	1.024	2.047
with Aflatoxin	7 days							
B1 and lactic								
acid 0.2%	After	ND	1.214 ±	0.333 ±	3.397 ±	1.443 ±	84.072 ±	168.144 ±
	pasteurization		0.005	0.006	0.006	0.040	0.343	0.686

LAB: Lactic acid bacteria (Log CFU/g), TA: Titratable acidity (% (w/v))

LA: Lactic acid (% (w/v)), AC: Acetic acid (% (w/v)).

4.5 References

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CHAPTER V

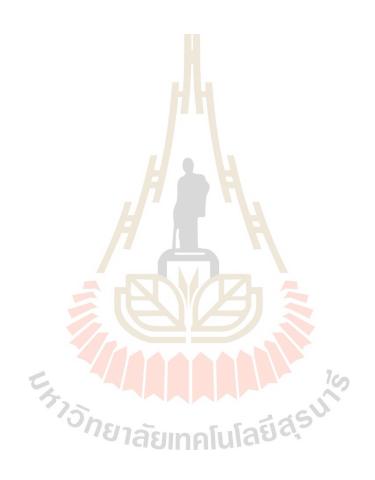
SUMMARY

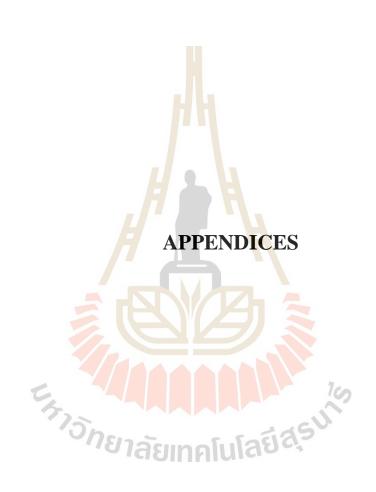
As mentioned previously, the heat resistant strain of spore forming bacteria *B. cereus* was considered to be the highest microbiological risk hazard in sauerkraut product. The process control measure must be evaluated for the ensuring of food safety as standard microbiological requirement. The cross-preventive factors, such as NaCl 1-5% (w/v), lactic acid 0.5-1.5% (w/v), and mild temperatures (65-85°C) for 20 min were performed to be the predictive microbiological model under Response Surface Methodology. The hurdle factors, NaCl 2% (w/v), lactic acid 1.2% (w/v), and pasteurization at 82°C for 20 min were validated being the limitation of control measure influencing the outgrowth of *B. cereus* spores and had damaged sub-letal spore targets, based on the combination criteria, they are spore survival, membrane fluidity, membrane permeability, DPA release, DNA release, and gene expression (*DnaK*, *hrc*A, *Abr*B and BC1006).

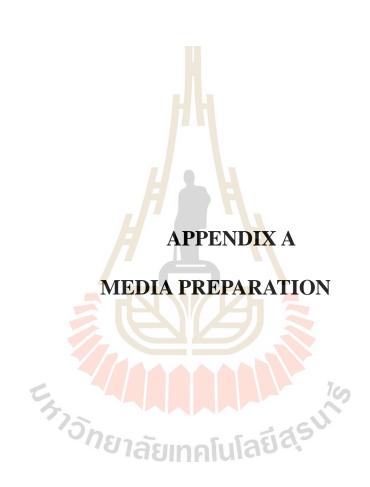
That of the limitation control point were verified under sauerkraut fermentation models which *B. cereus* spore and aflatoxin B1 were challenged. After 7 days of fermentation and ended with pasteurization, the viability of *B cereus* spore was not found and aflatoxin B1 was decreased (84.07%). In addition, bio-preservative agents from hetero-lactic fermentation, lactic and acetic acid have still remained.

Drawn from dissertation reveals that the cross-preventive stress factors, NaCl 2% (w/v), lactic acid 1.2% (w/v), and pasteurization at 82°C for 20 min could be

prototype model of control measurement for ensuring the safety of *B. cereus* and aflatoxin B1 cross-contamination during sauerkraut production. Meanwhile, this condition might be the implied development for Sanitation Standard Operating Procedures (SSOP) practice for further interest of fermented vegetable industry.







1A Plate count agar (PCA)

Pancreatic digest of casein 5 g 2.5 g Yeast extract 1 g Dextrose Agar 15 g Add distilled water to bring volume up to 1000 ml pH 7.0±0.2 The medium was autoclaved at 121°C for 15 min.

2A Nutrient agar (NA)

Peptone	5 g
Yeast extract	3 g
Agar	15 g
Add distilled water to bring volume up to	1000 ml
pH 7.0±0.2	
The medium was autoclaved at 121°C for 15 min.	
775na = ==================================	
3A Nutrient broth (NB) 81 as In Alufa 8 as	

Peptone	5 g
Yeast extract	3 g
Add distilled water to bring volume up to	1000 ml
pH 7.0±0.2	
The medium was autoclaved at 121°C for 15 min.	

4A Potato dextrose broth (PDB)

Potato starch 4 g 20 g Dextrose 1000 ml Add distilled water to bring volume up to pH 5.2±0.2 The medium was autoclaved at 121°C for 15 min.

5A Potato dextrose Agar (PDA)

Potato starch	4 g
Dextrose	20 g
Agar	15 g
Add distilled water to bring volume up to	1000 ml
pH 5.6±0.2	
The medium was autoclaved at 121°C for 15 min.	

6A De Man, Rogosa and Sharpe agar (MRS)	
Peptone Pepton	10 g
Beef extract	10 g
Yeast extract	4 g
Glucose	20 g
Sodium acetate trihydrate	5 g
Polysorbate 80 (also known as Tween 80)	1 g
Dipotassium hydrogen phosphate	2 g
Triammonium citrate	2 g

Magnesium sulfate heptahydrate	0.2 g
Manganese sulfate tetrahydrate	0.05 g
Agar	10 g
Add distilled water to bring volume up to	1000 ml
pH 6.2±0.2	

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

7A Mannitol Egg Yolk Polymyxin Agar (MYP)

Meat extract	1.0 g
Peptone	10 g
Mannitol	10 g
Sodium chloride	10 g
Phenol Red	0.025 g
Agar	12g
Add distilled water to bring volume up to	1000 ml
pH 7.2±0.2	

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

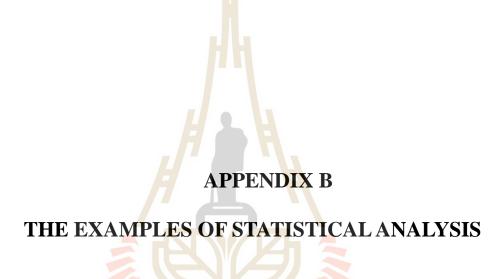


Table 1B. Effect of cross preventive factors, temperatures (65°C, 75°C, 85°C for 20 min), NaCl (1%, 3%, 5% (w/v)) and lactic acid (0.5, 1.0, 1.5% (w/v)) on spore germination rate (%), spore membrane permeability (% crystal violet up take), spore membrane fluidity (fluorescence intensity), DPA release (%) and DNA release (ng/μl) of *B. cereus* ATCC 11778 spore.

No.	Treatment		ent	Spore	Membrane	Membrane				
	LA (%)	NaCl (%)	Temp (°C)	germination rate (%)	permeability (% crystal violet uptake)	fluidity (fluorescence intensity)	DPA release (%)	DNA release (ng/μl)		
1	Untre	treatd		reatd		100.000 ± 0.000^{a}	15.917 ± 0.042^{a}	4.413 ± 0.159 ^a	100.000 ± 0.000^{g}	5.437 ± 0.032^{a}
2	0.5	1	65	86.937 ± 0.075^{t}	31.840 ± 0.195 ^b	20.490 ± 0.195^{b}	99.627 ± 0.283 ^g	28.303 ± 0.508^{b}		
3	0.5	3	65	74.670 ± 0.433^{s}	37.803 ± 0.092^{c}	33.367 ± 0.404°	99.737 ± 0.302 ^g	35.420 ± 0.346^{c}		
4	0.5	5	65	50.767 ± 0.276^{q}	40.743 ± 0.142°	37.490 ± 0.195^{e}	99.430 ± 0.793 ^g	60.383 ± 0.357^{e}		
5	1	1	65	$60.867 \pm 0.080^{\text{r}}$	$39.660 \pm 0.154^{\mathbf{d}}$	36.603 ± 0.291^{d}	32.130 ± 1.462^{a}	$40.543 \pm 0.306^{\mathbf{d}}$		
6	1	3	65	37.760 ± 0.131 ^p	$41.250 \pm 0.632^{\text{ef}}$	$40.333 \pm 0.260^{\mathbf{f}}$	32.443 ± 1.440^{a}	$78.810 \pm 0.701^{\mathbf{f}}$		
7	1	5	65	33.580 ± 0.272°	$41.633 \pm 0.185^{\text{fg}}$	41.683 ± 0.175^{g}	37.850 ± 1.338 ^b	84.413 ± 0.416^{g}		
8	1.5	1	65	$22.263 \pm 0.075^{\mathbf{j}}$	46.787 ± 0.387^{k}	48.557 ± 0.301^{k}	35.163 ± 1.476^{ab}	127.580 ± 0.319^{1}		
9	1.5	3	65	21.683 ± 0.227^{i}	47.473 ± 0.110^{1}	48.820 ± 0.815^{1}	38.533 ± 0.962^{b}	$128.350 \pm 0.395^{\mathbf{m}}$		
10	1.5	5	65	$13.697 \pm 0.253^{\mathrm{g}}$	51.310 ± 0.333^{n}	$55.167 \pm 0.060^{\text{n}}$	47.593 ± 2.864^{c}	133.620 ± 0.352°		
11	0.5	1	75	28.343 ± 0.115 ⁿ	42.107 ± 0.251^{g}	42.453 ± 0.319 ^h	99.423 ± 0.295^{g}	$100.410 \pm 0.422^{\mathbf{h}}$		
12	0.5	3	75	$27.390 \pm 0.305^{\text{m}}$	43.757 ± 0.232 ^h	43.273 ± 0.240^{i}	99.027 ± 0.590^{g}	120.400 ± 0.081^{i}		
13	0.5	5	75	25.753 ± 0.218^{1}	44.807 ± 0.436^{i}	44.507 ± 0.260^{j}	99.380 ± 0.568^{g}	$125.140 \pm 0.100^{\mathbf{j}}$		
14	1	1	75	15.810 ± 0.104^{h}	$50.267 \pm 0.808^{\rm n}$	$51.197 \pm 0.133^{\text{m}}$	$74.167 \pm 6.052^{\mathbf{d}}$	$130.430 \pm 0.252^{\text{n}}$		
15	1	3	75	$4.920 \pm 0.072^{\mathbf{d}}$	58.227 ± 0.319^{q}	65.187 ± 0.140^{q}	78.907 ± 0.588^{e}	$138.340 \pm 0.168^{\mathbf{r}}$		
16	1	5	75	0.443 ± 0.012^{b}	63.130 ± 0.312^{s}	$70.427 \pm 0.191^{\rm s}$	80.873 ± 0.515^{e}	145.830 ± 0.234^{t}		
17	1.5	1	75	11.737 ± 0.308^{e}	53.293 ± 0.110°	59.367 ± 0.404°	47.593 ± 2.864^{c}	$134.770 \pm 0.150^{\mathbf{p}}$		
18	1.5	3	75	0.307 ± 0.006^{ab}	63.267 ± 0.144^{s}	72.550 ± 0.053^{t}	47.650 ± 1.445^{c}	$148.560 \pm 0.367^{\mathrm{u}}$		
19	1.5	5	75	0.090 ± 0.000^{a}	64.060 ± 0.279^{t}	$74.410 \pm 0.020^{\mathbf{u}}$	48.653 ± 3.918°	$150.640 \pm 0.183^{\mathrm{v}}$		
20	0.5	1	85	$24.640 \pm 0.069^{\mathbf{k}}$	45.493 ± 0.297^{j}	45.220 ± 0.046^{k}	99.363 ± 0.512^{g}	126.590 ± 0.122^{k}		
21	0.5	3	85	6.823 ± 0.121^{e}	56.777 ± 0.098^{p}	$63.360 \pm 0.173^{\text{p}}$	99.693 ± 0.557^{g}	137.110 ± 0.111^{q}		
22	0.5	5	85	0.000 ± 0.000^{a}	$64.857 \pm 0.837^{\mathrm{u}}$	$76.887 \pm 0.206^{\text{y}}$	99.177 ± 0.480^{g}	$151.560 \pm 0.372^{\text{v}}$		
23	1	1	85	2.127 ± 0.031^{c}	59.680 ± 0.288^{r}	$68.490 \pm 0.195^{\mathbf{r}}$	79.913 ± 0.677^{e}	$142.470 \pm 0.226^{\text{s}}$		
24	1	3	85	0.010 ± 0.000^{a}	$64.603 \pm 0.326^{\text{tu}}$	76.850 ± 0.052^{v}	80.937 ± 0.215^{e}	$151.520 \pm 0.055^{\text{w}}$		
25	1	5	85	0.000 ± 0.001^{a}	$64.857 \pm 0.530^{\mathrm{u}}$	$76.877 \pm 0.110^{\mathbf{w}}$	$86.003 \pm 3.298^{\mathbf{f}}$	$151.560 \pm 0.087^{\mathbf{w}}$		
26	1.5	1	85	0.000 ± 0.002^{a}	$64.800 \pm 0.193^{\mathrm{u}}$	76.890 ± 0.101^{z}	38.557 ± 1.197 ^b	$151.560 \pm 0.184^{\mathbf{w}}$		
27	1.5	3	85	0.000 ± 0.003^{a}	$64.853 \pm 0.427^{\mathrm{u}}$	76.887 ± 0.107^{x}	74.300 ± 5.925^{d}	$151.560 \pm 0.285^{\text{w}}$		
28	1.5	5	85	0.000 ± 0.004^{a}	$64.800 \pm 0.193^{\mathrm{u}}$	$76.887 \pm 0.105^{\text{y}}$	81.273 ± 0.219^{e}	$151.820 \pm 0.064^{\text{w}}$		

The different letter, a, b, c, ..., and w between groups indicate significant differences at p<0.05.

Table 2B. Coded and actual levels of independent variables A. lactic acid (% (w/v)),B. NaCl (% (w/v)), C. temperature (°C) used in central composite design along with measured responses.

Run		ectic l (%)		ıCl %)	Tempe		Spore germination rate (%)	Membrane permeability (% of crystal violet uptake)	Membrane fluidity (fluorescence intensity)	DPA release (%)	DNA release (ng/µl)
•	C	A	C	A	С	A	•	арчан е)			
1	-1	0.5	1	5	1	85	0	64.86	76.89	88.23	151.56
2	-1	0.5	1	5	0	75	25.75	44.81	44.51	68.71	125.14
3	0	1	-1	1	1	85	2.13	59.68	68.49	84.27	142.47
4	1	1.5	1	5	1	85	0	64.8	76.89	88.22	151.82
5	1	1.5	1	5	-1	65	13.7	51.31	55.17	76.27	133.62
6	0	1	0	3	0	75	4.92	58.23	65.19	83.7	138.34
7	1	1.5	1	5	0	75	0.09	64.06	74.41	87.75	150.64
8	0	1	1	5	0	75	0.04	63.13	70.43	86.44	145.83
9	1	1.5	-1	1	-1	65	22.26	46.79	48.56	73.36	127.58
10	-1	0.5	-1	1	-1	65	86.94	31.84	20.49	33.56	28.3
11	0	1	0	3	-1	65	37.76	41.25	40.33	60.46	78.81
12	0	1	-1	1	-1	65	60.87	39.66	36.6	54.5	40.54
13	-1	0.5	-1	1	1	85	24.64	45.59	45.52	69.71	126.59
14	-1	0.5	0	3	1	85	6.82	56.78	63.36	83.18	137.11
15	1	1.5	-1	1	1	85	0	64.8	76.89	88.22	151.56
16	0	1	1	5	1	85	0	64.86	76.88	88.22	151.56
17	-1	0.5	0	3	0	75	27.39	43.76	43.27	66.54	120.4
18	0	1	1	5	-1	65	33.58	41.63	41.68	64.5	84.41
19	-1	0.5	-1	1	0	75	28.34	42.11	42.25	65.76	100.41
20	1	1.5	-1	1	0	75	11.74	53.29	59.37	81.53	134.77
21	-1	0.5	1	5	-16	65	50.77	40.74	37.49	58.89	60.38
22	1	1.5	0	3	0	75	0.31	63.27	72.55	86.7	148.56
23	0	1	0	3	1	85	0.01	64.6	76.85	88.18	151.52
24	1	1.5	0	3	-1	65	21.68	47.47	48.82	74.75	128.35
25	0	1	-1	1	0	75	15.81	50.27	51.2	75.62	130.43
26	1	1.5	0	3	1	85	0	64.85	76.89	88.23	151.56
27	-1	0.5	0	3	-1	65	74.67	37.8	33.37	35.51	35.42

C and A are coded and actual level, respectively.

Table 3B. Design summary and estimated regression coefficients for dependent variables and their significance.

Factor	Spore germination rate (%)	Membrane permeability (% of crystal violet uptake)	Membrane fluidity (fluorescence intensity)	DPA release (%)	DNA release (ng/μl)
Model	Quadratic significant	Quadratic significant	Quadratic significant	Quadratic significant	Quadratic significant
Intercept	8.470**	55.862**	61.692**	80.209**	132.968**
A	14.200**	6.242**	10.133**	9.719**	21.842**
В	7.160**	3.676**	5.832**	4.483**	9.573**
C	20.480**	9.574**	15.342**	13.037**	33.241**
AB	3.600*	-1.298	-2.415	-3.139*	-4.968
AC	10.270**	-0.664	-1.358	-6.073**	-18.814**
BC	3.770*	0.755	0.923	-1.314	-3.973
A2	4.71	-2.094	-3.256	-3.148	1.997
B2	1.64	-1.211	-2.086	-0.041	-2.363
C2	11.500**	-2.031	-2.511	-5.958**	-19.771**
R2	96.3	92.06	93.56	95.82	95.48
Lack of fit	Significant	Significant	Significant	Significant	Significant

^{**} and * between groups indicate significant differences at p<0.01 and p<0.05, respectivetly.

4B. Final Equation in Terms of Coded Factors and Actua factor of responses

- Final Equation in Terms of Coded Factors:

Spore germination rate (%) = $+(9.57) - (14.20*A) - (7.16*B) - (20.48*C) + (3.60*A*B) + (10.27*A*C) + (3.77*B*C) + (4.71*(A)^2) + (11.50*(C)^2)$

Membrane permeability (% of crystal violet uptake) = $+ (55.86) + (6.24*A) + (3.68*B) + (9.57*C) - (1.30*A*B) - (0.66*A*C) + (0.76*B*C) - (2.09*(A)^2) - (1.21*(B)^2) - (2.03*(C)^2)$

Membrane fluidity (fluorescence intensity) = $+ (61.69 + (10.13*A) + (5.83*X2) + (15.34*C) - (2.41*A*X2) - (1.36*A*C) + (0.92*X2*C) - (3.26*(A)^2) - (2.09*(X2)^2) - (2.51*(C)^2)$

DPA release (%) =
$$+(80.21) + (9.72*A) + (4.48*X2) + (13.04*C) - (3.14*A*X2) - (6.07*A*C) - (1.31*X2*C) - (3.15*(A)^2) - (0.041*(X2)^2) - (5.96*(C)^2)$$

DNA release
$$(ng/\mu l) = + (132.97) + (21.84*A) + (9.57*X2) + (33.24*C) - (4.97*A*X2) - (18.81*A*C) - (3.97*X2*C) + (2.00*(A)2) - (2.36*(X2)2) - (19.77*(C)2)$$

- Final Equation in Terms of Actua factor:

Spore germination rate (%) = + (1075.49794) - (231.00639*Lactic acid) - (21.31757*NaCl - 21.92240*Temperature) + (3.59917*Lacticacid*NaCl) + (2.05467*Lacticacid*Temperature) + (0.18854*NaCl*Temperature) + (18.85778*((Lacticacid)^2)+ (0.11503*(Temperature)^2)

Membrane permeability (% of crystal violet uptake) = -164.62162 + 43.09194*Lactic acid + 2.12097*NaCl + 4.02281*Temperature - 1.29833*Lactic acid*NaCl - 0.13283*Lactic acid*Temperature + 0.037750*NaCl*Temperature - 8.37556*(Lactic acid)² - 0.30264*(NaCl)² - 0.020306*(Temperature)²

Membrane fluidity (fluorescence intensity) = $-(258.56100) + (73.93111*Lactic acid) + (5.00007*NaCl) + (5.43329*Temperature) - (2.41500*Lactic acid*NaCl) - (0.27167*Lactic acid*Temperature) + (0.046125*NaCl*Temperature) - (13.02222*(Lactic acid)^2) - (0.52139*(NaCl)^2) - (0.025106*(Temperature)^2)$

DPA release (%) = - (506.82650) + (145.12500*Lactic acid) + (10.37062*NaCl) + (11.65196*Temperature) - (3.13917*Lactic acid*NaCl) - (1.21450*Lactic acid*Temperature) - (0.065708*NaCl*Temperature) - $(12.59111*(Lactic acid)^2)$ - $(0.010278*(NaCl)^2)$ - $(0.059578*(Temperature)^2)$

DNA release $(ng/\mu l) = -1625.64067 + 324.82056*Lactic acid + 28.19493*NaCl + 37.33949*Temperature - 4.96750*Lactic acid*NaCl - 3.76283*Lactic acid*Temperature - 0.19862*NaCl*Temperature + 7.98889*(Lactic acid)^2 - 0.59069*(NaCl)^2 - 0.19771*(Temperature)^2$



APPENDIX C

DnaK, hrcA, AbrB, BC006 GENES, PARTIAL CDS,

GENBANK

1C. DnaK, gene, partial cds, GenBank: NC_004722

LOCUS NC_004722 1836 bp DNA linear CON 28-AUG-2016

DEFINITION Bacillus cereus ATCC 14579 chromosome, complete genome.

ACCESSION NC_004722

VERSION NC_004722.1

KEYWORDS RefSeq.

SOURCE Bacillus cereus ATCC 14579

ORGANISM Bacillus cereus ATCC 14579

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;

Bacillus cereus group.

REFERENCE 1 (bases 1 to 1836)

AUTHORS Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B.,

Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N.,

Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N.,

D'Souza,M.,

Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R., Fonstein, M.,

Ehrlich, S.D., Overbeek, R. and Kyrpides, N.

TITLE Genome sequence of *Bacillus cereus* and comparative analysis with

Bacillus anthracis

JOURNAL Nature 423 (6935), 87-91 (2003)

PUBMED 12721630

REFERENCE 2 (bases 1 to 1836)

AUTHORS Candelon, B., Gailloux, K., Ehrlich, D.S. and Sorokin, A.

TITLE The number of ribosomal RNA operons in *Bacillus cereus*

JOURNAL Submitted (10-SEP-2004) National Center for Biotechnology

Information, NIH, Bethesda, MD 20894, USA

FEATURES Location/Qualifiers

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CDS 1..1836

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2C. BC1006, gene, partial cds, GenBank: NC_004722

LOCUS NC 004722 1143 bp DNA linear CON 28-AUG-2016

DEFINITION Bacillus cereus ATCC 14579 chromosome, complete genome.

ACCESSION NC_004722 REGION: 985909..987051

VERSION NC_004722.1

KEYWORDS RefSeq.

SOURCE Bacillus cereus ATCC 14579

ORGANISM <u>Bacillus cereus ATCC 14579</u>

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;

Bacillus cereus group.

REFERENCE 1 (bases 1 to 1143)

AUTHORS Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B.,

Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N.,

Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N.,

D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R.,

Fonstein, M., Ehrlich, S.D., Overbeek, R. and Kyrpides, N.

TITLE Genome sequence of *Bacillus cereus* and comparative analysis with

Bacillus anthracis

JOURNAL Nature 423 (6935), 87-91 (2003)

PUBMED <u>12721630</u>

REFERENCE 2 (bases 1 to 1143)

AUTHORS Candelon, B., Gailloux, K., Ehrlich, D.S. and Sorokin, A.

TITLE The number of ribosomal RNA operons in *Bacillus cereus*

JOURNAL Submitted (10-SEP-2004) National Center for Biotechnology

Information, NIH, Bethesda, MD 20894, USA

FEATURES Location/Qualifiers

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CDS 1..1143

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ORIGIN

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3C. hrcA, gene, partial cds, GenBank: NC_004722

LOCUS NC 004722 1017 bp DNA linear CON 28-AUG-2016

DEFINITION Bacillus cereus ATCC 14579 chromosome, complete genome.

ACCESSION NC_004722 REGION: complement (4256576..4257592)

VERSION NC_004722.1

KEYWORDS RefSeq.

SOURCE Bacillus cereus ATCC 14579

ORGANISM <u>Bacillus cereus ATCC 14579</u>

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;

Bacillus cereus group.

REFERENCE 1 (bases 1 to 1017)

AUTHORS Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B.,

Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N.,

Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N.,

D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R.,

Fonstein, M., Ehrlich, S.D., Overbeek, R. and Kyrpides, N.

TITLE Genome sequence of *Bacillus cereus* and comparative analysis with

Bacillus anthracis

JOURNAL Nature 423 (6935), 87-91 (2003)

PUBMED <u>12721630</u>

REFERENCE 2 (bases 1 to 1017)

AUTHORS Candelon, B., Gailloux, K., Ehrlich, D.S. and Sorokin, A.

TITLE The number of ribosomal RNA operons in *Bacillus cereus*

JOURNAL Submitted (10-SEP-2004) National Center for Biotechnology

Information, NIH, Bethesda, MD 20894, USA

FEATURES Location/Qualifiers

source 1..1017

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/mol_type="genomic DNA"

/strain="ATCC 14579"

/db_xref="ATCC:<u>14579</u>"

/db_xref="taxon:226900"

gene 1..1017

/gene="hrcA"

/locus_tag="BC4314"

/db_xref="GeneID:1206659"

CDS 1..1017

/gene="hrcA"

/locus_tag="BC4314"

/note="Negative regulator of class I heat shock genes

(grpE-dnaK-dnaJ and groELS operons). Prevents heat-shock

induction of these operons"

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AIVLGPKLSTNKLKNVQIVPLDRQTAVAIIVTDTGHVQSKTIT
VPESVDLSDLEKMVNILNEKLSGVPMEELHNKIFKEIVTVLR
GYVHNYDSAIKMLDGTFQVPLSEKIYFGGKANMLSQPEFH
DIQKVRSLLTMIDNEAEFYDILRHKQVGIQVKIGRENSSTAM
EDCSLISATYSIGEEQLGTIAILGPTRMQYSRVISLLQLFTRQF
TDGLKK"

ORIGIN

1 atgcttacgg aacgtcagct cttaatttta caaacaatta ttgatgactt tattggatca 61 gcgcagcccg ttgggtctag aacgttggct aaaaaagatg aaatcacatt tagttcagct 121 actattcgaa atgaaatggc ggacttagaa gaattaggct ttattgaaaa aacgcacagt 181 tettetggae gtgtteette tgagaaagge taeegatttt atgtagaeea tettttageg 241 ccgcaaaact taccgaa<mark>cgc</mark> tgaaatt<mark>gta</mark> caaattaaag atttatttgc tgaaagaatt 301 tttgaagegg aaaaaattge acageaatet geteaaattt tateagaact taegaattat 361 acggcaattg ttcttggacc gaagttaagt acaaataaac ttaaaaatgt acaaattgtg 421 ccgcttgatc gtcaaactgc agtcgctatt attgtaaccg atacagggca tgtacaaagt 481 aaaacgatta ccgttccgga atctgttgat ttatcagatt tagaaaaaaat ggttaatatt 541 ttaaatgaaa agctatctgg cgtaccgatg gaagaacttc ataataaaat ctttaaggaa 601 attgttacag ttttacgtgg gtatgttcat aattacgata gtgcaataaa aatgttggat 661 ggtacatttc aagttccgtt atcggaaaag atatactttg gaggaaaagc aaatatgctt 721 tegeagecag agttecatga catteaaaag gttagatett taettaceat gattgataat 781 gaageegaat tttatgacat tttgegteat aaacaagteg ggatteaagt gaaaattggt 841 agggaaaact ettegaegge tatggaggat tgtagtttaa tttetgeaac atattegate 901 ggcgaagagc aacttggaac aattgctatt ttaggtccta cgagaatgca atactctcgt 961 gtaattagtt tgttacagtt atttacgaga caatttactg atgggcttaa aaagtaa //

4C. AbrB, gene, partial cds, GenBank: NC_003997

LOCUS NC 003997 285 bp DNA linear CON 06-OCT-2017

DEFINITION Bacillus anthracis str. Ames chromosome, complete genome.

ACCESSION NC_003997 REGION: complement(40823..41107)

VERSION NC_003997.3

KEYWORDS RefSeq.

SOURCE Bacillus anthracis str. Ames

ORGANISM Bacillus anthracis str. Ames

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;

Bacillus cereus group.

REFERENCE 1 (bases 1 to 285)

AUTHORS Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., Paulsen, I.T.,

Nelson, K.E., Tettelin, H., Fouts, D.E., Eisen, J.A., Gill, S.R.,

Holtzapple, E.K., Okstad, O.A., Helgason, E., Rilstone, J., Wu, M.,

Kolonay, J.F., Beanan, M.J., Dodson, R.J., Brinkac, L.M., Gwinn, M.,

DeBoy, R.T., Madpu, R., Daugherty, S.C., Durkin, A.S., Haft, D.H.,

Nelson, W.C., Peterson, J.D., Pop, M., Khouri, H.M., Radune, D.,

Benton, J.L., Mahamoud, Y., Jiang, L., Hance, I.R., Weidman, J.F.,

Berry, K.J., Plaut, R.D., Wolf, A.M., Watkins, K.L., Nierman, W.C.,

Hazen, A., Cline, R., Redmond, C., Thwaite, J.E., White, O.,

Salzberg, S.L., Thomason, B., Friedlander, A.M., Koehler, T.M.,

Hanna, P.C., Kolsto, A.B. and Fraser, C.M.

TITLE The genome sequence of Bacillus anthracis Ames and comparison to

closely related bacteria

JOURNAL Nature 423 (6935), 81-86 (2003)

PUBMED 12721629

REFERENCE 2 (bases 1 to 285)

AUTHORS Joardar, V., Shrivastava, S., Brinkac, L.M., Harkins, D.M., Durkin, A.S.

and Sutton,G.

TITLE Direct Submission

JOURNAL Submitted (19-OCT-2009) The J. Craig Venter Institute, 9704

Medical Center Dr, Rockville, MD 20850, USA

FEATURES Location/Qualifiers

source 1..285

/organism="Bacillus anthracis str. Ames"

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/db_xref="taxon:198094"

gene 1..285

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/db_xref="Pathema:BA_0034"

CDS 1..285

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/old_locus_tag="BA0034"

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ORIGIN

1 atgaaateta etggtategt tegtaaagtt gatgaattag gtegtgtagt aatteeaate

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121 gagaaaatca tettaaaaaa atataaacca aacatgactt gecaagtaac tggtgaagta

181 tetgatggta acctattttt agetgaaggt aaaattatet taagcaaaga aggegetgag

241 caaatettaa acgaacttea agattatate gaaacagcaa aataa

รักยาลัยเทคโนโลยีสุรูนาง

//

APPENDIX D HPLC CHROMATOGRAM AND STANDARD CURVE OF LACTIC ACID AND ACETICACID

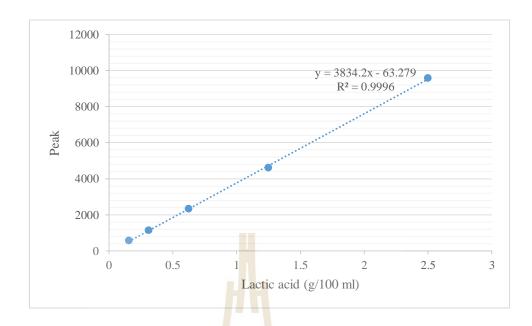


Figure 1D. Calibration curve of lactic acid

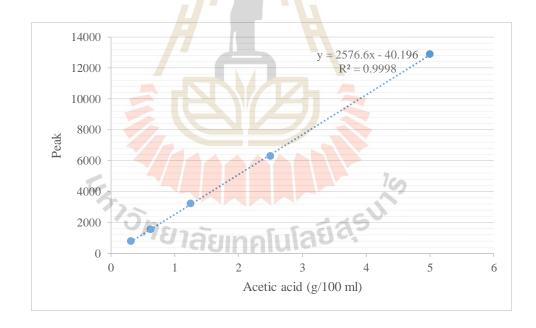


Figure 2D. Calibration curve of acetic acid

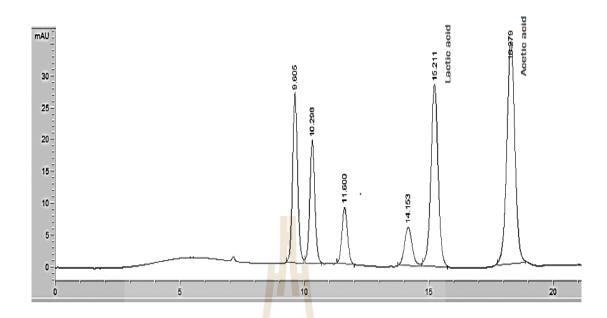


Figure 3D. HPLC chromatogram of lactic acid (retention time 15.211) and acetic acid (retention time 18.979) standard was at 205nm.

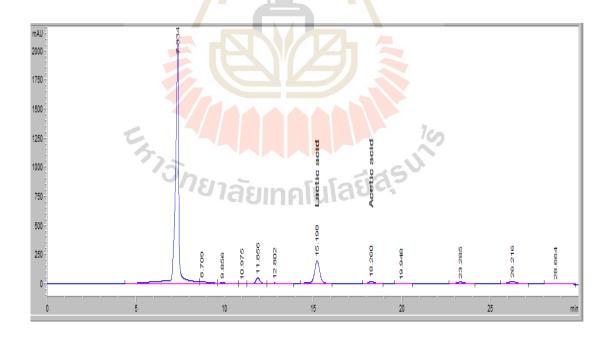


Figure 4D. HPLC chromatogram of lactic acid and acetic acid brine sauerkraut fermentation was at 205nm.



FOURIER TRANSFORM INFRARED SPECTROSCOPY

(FTIR) SPECTRA

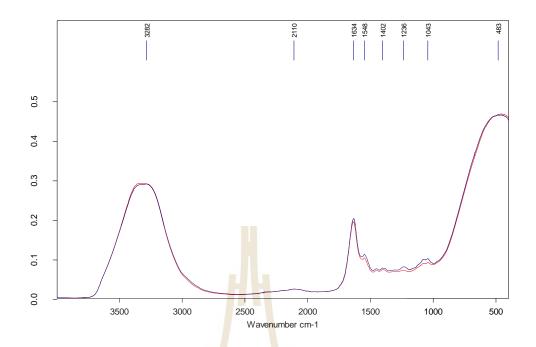


Figure 1E. FTIR spectra of fresh spore (blue) and NaCl 2% (w/v) treated spore (red).

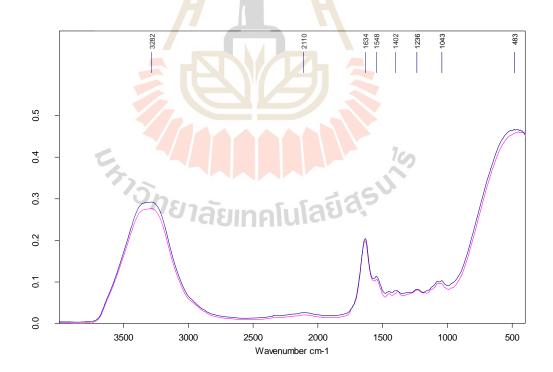


Figure 2E. FTIR spectra of fresh spore (blue) and lactic acid 2% (w/v) treated spore (pink).

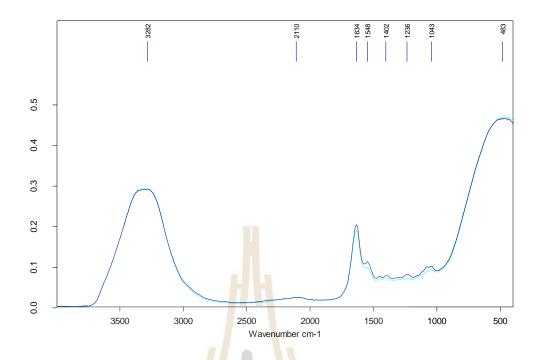


Figure 3E. FTIR spectra of fresh spore (blue) and temperature 82°C for 20 min treated spore (green).

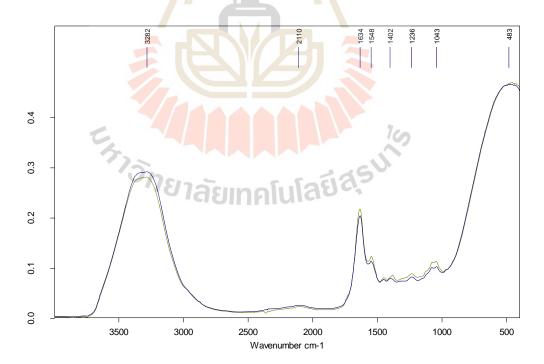


Figure 4E. FTIR spectra of fresh spore (blue) and lactic acid 2% (w/v) and NaCl 2% (w/v) treated spore (yellow green).

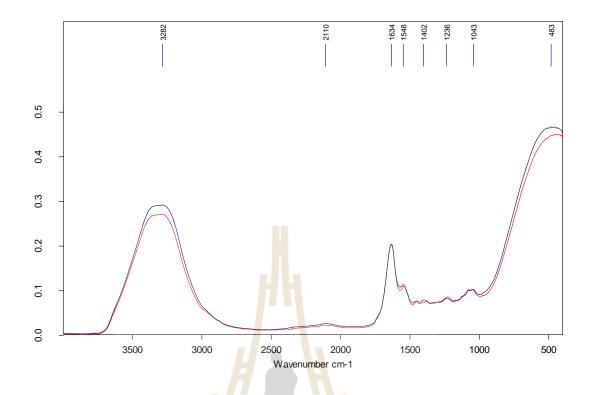


Figure 5E. FTIR spectra of fresh spore (blue) and combination of lactic acid 2% (w/v), NaCl 2% (w/v), and temperature 82°C for 20 min treated spore (red).



BIOGRAPHY

Nguyen Thi Ngoc Huong was born on 19th September 1982 in Quangnam province, Vietnam. She received a Bachelor of Engineering in Food Technology degree from Danang University of Technology, Vietnam in 2006, and a Master Degrees in Food and Beverage Technology from Danang University, Vietnam in 2011. She obtains a Degree of Doctor of Philosophy in Food Technology studies in academic year 2017 from School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. She is currently a lecturer at the Faculty of Food Technology, College of Food Industry, Danang city, Vietnam. Her academic areas of interest mainly in Food fermentation, Food microbiology, Food safety, and Food quality assurance.

Poster presentation:

Nguyen, T. N. H and Gasaluck, P. (2016). The influence of pasteurization control factors on surrogate *Aspergillus* sp. in sauerkraut model. **The 4th SUT**International Agricultural Colloquium.