FOURIER TRANSFORM INFRARED SPECTROSCOPY FOR RAPID DETECTION OF BACTERIAL SPOILAGE IN MILK

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เทคนิคฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปีเพื่อการตรวจสอบการเน่าเสียที่เกิดจากแบคทีเรียในนมแบบรวดเร็ว

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FOURIER TRANSFORM INFRARED SPECTROSCOPY FOR RAPID DETECTION OF BACTERIAL SPOILAGE IN MILK

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

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อาจารย์ที่ปรึกษา รองศาสตราจารย์ ดร ทัศนีย์ เสาวนะ, 81 หน้า.

วัตถุประสงค์ของงานวิจัยนี้ คือ ศึกษาความเป็นไปได้ในการวิเคราะห์ปริมาณเชื้อแบคทีเรียที่ทำให้เกิดการเน่าเสียของนมแบบรวดเร็ว โดยตรวจวัดสเปกตรัมขององค์ประกอบของนมที่เปลี่ยนแปลงไป ด้วยเครื่องฟูเรียร์ทรานสฟอร์มอินฟราเรด (เอฟที-ไออาร์) ไมโครสเปกโทรสโกปีแบบเครื่องตรวจจับอาเรย์ระนาบโฟกัส ช่วงเลขคลื่น 4000-400 ซม.$^{-1}$ และนำเชื้อแบคทีเรีย *B. cereus* 11778 และ *P aeruginosa* ATCC 15442 มาเติมในตัวอย่างนมยูเอชทีทั้งแบบเดี่ยวและผสม จากนั้นนำไปบ่มในตู้บ่มแบบเขย่าด้วยความเร็ว 200 rpm ช่วงเวลา 24 ชั่วโมงที่อุณหภูมิ 37 องศาเซลเซียส นำตัวอย่างที่ได้มาตรวจวิเคราะห์จำนวนแบคทีเรีย ควบคู่ไปกับการตรวจวัดสเปกตรัมของตัวอย่างนมในช่วงเลขคลื่น 3000-2800 cm^{-1} ของกรดไขมัน 1800-1500 cm^{-1} ของโปรตีน และ 1200-900 cm^{-1} ของโพลีแซกคาไรด์ จากนั้นนำข้อมูลสเปกตรัมแบบการดูดกลืนแสงที่ผ่านการจัดการทางคณิตศาสตร์ด้วยวิธีอนุพันธ์ ที่สอง นำไปสร้างแบบจำลองในการทำนายปริมาณเชื้อแบคทีเรียด้วยการวิเคราะห์การถดถอย ด้วยวิธีกำลังสองน้อยที่สุดบางส่วน (Partial least square, PLS) โดยแบบจำลองในการทำนายปริมาณเชื้อที่ดีที่สุด มีค่าสัมประสิทธิ์การตัดสินใจ (coefficient of determination; R^2) เท่ากับ 0.98 ค่าความผิดพลาดยกกำลังสองเฉลี่ยของการพิสูจน์แบบไขว้ (root mean squares error of the cross-validation; RMSECV) เท่ากับ 0.16 ความผิดพลาดเฉลี่ย (bias) เท่ากับ -0.000646 และ อัตราส่วนระหว่างค่าส่วนเบี่ยงเบนมาตรฐานของค่าอ้างอิงของชุดทำนายต่อค่าส่วนเบี่ยงเบนมาตรฐานของความผิดพลาดของชุดทำนาย (residual prediction deviation; RPD) เท่ากับ 7.35 จากค่า R^2 มากกว่าหรือเท่ากับ 0.95 นั้น แสดงถึงความสัมพันธ์เชิงเส้นที่ดีระหว่างค่าที่ทำนายได้จากเอฟที-ไออาร์ (FT-IR predicted) กับค่า จำนวนแบคทีเรียที่วัดได้จริง (actual TVC) สามารถนำมาไปใช้เป็นแนวทางสำหรับการตรวจการเน่าเสียของนมจากแบคทีเรียแบบรวดเร็วได้ ซึ่งมีความสัมพันธ์กับการเปลี่ยนแปลงองค์ประกอบของนมที่เกิดจากขบวนการเมแทบอลิซึม

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ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PONGPAN WATCHARAWICHANAN : FOURIER TRANSFORM INFRARED SPECTROSCOPY FOR RAPID DETECTION OF BACTERIAL SPOILAGE IN MILK. THESIS ADVISOR : ASSOC. PROF. TASSANEE SAOVANA, Ph.D. 81 PP.

BACTERIAL SPOILAGE/ FOCAL PLANE ARRAY, FT-IR/ RAPID DETECTION/ *BACILLUS CEREUS*/ *PSEUDOMONAS AERUGINOSA*

The objectives of this thesis were to investigate the feasibility on evaluation for rapid detection of bacterial spoilage in milk by a detected spectrum of compositional change of milk using Fourier Transform Infrared (FT-IR) microspectroscopy, focal plane array (FPA) detector (4000-900 cm^{-1}). *Bacillus cereus* ATCC 11778 and *Pseudomonas aeruginosa* ATCC 15442 were inoculated into the UHT whole milk with mono and co-culture. Inoculated milk samples were rotated at 200 revolutions per minute (rpm), during 24 hours (h) of growth at 37 degree Celsius (°C). The samples were determined the total viable counts (TVC), while the same samples were analysed the spectrum of milk from fatty acid region (3000-2800 cm^{-1}), proteins (1800-1500 cm^{-1}) and polysaccharides (1200-900 cm^{-1}). The absorbance spectra with mathematic pretreatment of the second derivative were developed the calibration model for total viable counts by partial least square (PLS). The calibration models provided the best model (coefficient of determination; (R^2) = 0.98, root mean squares error for the cross-validation (RMSECV) = 0.16, bias = -0.000646 and residual prediction deviation (RPD) = 7.35. Since R^2 was $\geq$ 0.95, PLS model was performed good linear relationship between FT-IR predicted value and actual TVC

value. It could be used as a guideline for rapid detection of bacterial spoilage in milk correlated with the compositional changes in milk derived from the metabolic activity.

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LIST OF ABBREVI ATIONS

°C	=	Degree Celsius
mg	=	Milligram
gm	=	Gram
µg	=	Microgram
µm	=	Micrometer
ml	=	Milliliter
rpm	=	Revolution per minute
ATCC	=	American Type Culture Collection
DMST	=	Department of Medical Sciences, Thailand
cfu	=	Colony Forming Unit
min	=	Minutes
h	=	Hours
OD	=	Optical Density
UV	=	Ultra Violet
VIS	=	Visible
PBS	=	Phosphate Buffer Saline
W/V	=	Weight by Volume
FT-IR	=	Fourier Transform Infrared Spectroscopy
FPA	=	Focal Plane Array
PCA	=	Principle Component Analysis

LIST OF ABBREVIATIONS (Continued)

ANOVA	=	Analysis of Variance
ATP	=	Adenosine Triphosphate
BC	=	*Bacillus cereus*
DTGS	=	Deuterated Triglycine Sulphate
EMSC	=	Extended Multiplication Signal Correction
ELISA	=	Enzyme-Linked Immunofluorescence
FITC	=	Fluorescein Isothiocyanate
IF	=	Immonofluorescence
MALDI-TOF-MS	=	Matrix-assised laser desorption/ionization-time-of-flight-Mass spectroscopy
MCT	=	Mercury Cadmium Tellurite
Mg^{2+}	=	Magnesium ion
PA	=	*Pseudomonas aeruginosa*
PLS	=	Partial Least Squares
Ppb	=	Part per billion
R^2	=	Determination coeffient
RPD	=	Residual Prediction Deviation
RMSC	=	Root Mean Square Error of Cross-validation
RT-PCR	=	Real-Time Polymerase Chain Reaction
SD	=	Standard Deviation
SEM	=	Standard Error of Means

LIST OF ABBREVIATIONS (Continued)

SNR	=	Signal-to-Noise Ratio
TySA	=	Tyramide-Signal Amplification

CHAPTER I

INTRODUCTION

1.1 Introduction

Milk is the first food for young mammals, including human infants and children. It is the particular constituent of the diet because it contains a highly nutritious nature and it has calcium which is essential for the formation and maintenance of bones and teeth. Even though the major constituent of milk is water, but according to species, milk contains varying quantities of lipids, proteins, and carbohydrates synthesised within the mammary gland and smaller amounts of minerals and other fat-soluble and water-soluble components derived directly from blood plasma (Varnam and Sutherland, 2001). Besides mother's milk, the dairy products for human consumption are produced by cows, buffaloes, sheep, goats, and camels. Cow's milk is much favourable for the world's population and is the vast majority of the processed milk which mostly is the pasteurised milk. Thus, the dairy industry must concern about the microbial quality of milk since pasteurised milk may not destroy all foodborne pathogens in milk. The dominant pathogens are *Campylobacter jejuni*, *Listeria monocytogenes*, *Salmonella* spp., and Shiga toxin–producing *Escherichia coli* (STEC). These bacteria can survive and grow in post-pasteurisation processing, leading to the contaminated milk. For this reason, it is a risk to the consumer from direct exposure to foodborne pathogens presented in dairy products that come from re-contaminated after pasteurisation (Oliver et al., 2005). In these recent years, it is

increase dramatically to concern about safety food production because it directly affects to human's health. The significant variation of products and low consumer loyalty including products spoilt by microbiological growth have a large impact on existing accepted products and on new products present into the markets. (Rysstad and Kolstad, 2006)

The spoilage dairy products which can be detected by organoleptic changes are unacceptable to consumers. These organoleptic taints are visible, bad odours and bad flavours. (Nicolaou and Goodacre, 2008). The worried spoilage bacteria are aerobic psychrotrophic gram-negative bacteria, heterofermentative lactobacilli, and spore-forming bacteria. Psychrotrophic bacteria can produce many extracellular hydrolytic enzymes and be the cause of the pasteurised milk recontamination which is a fundamental element of its shelf life (Ledenbach and Marshall, 2009). The types of organoleptic changes vary according to the species of the bacteria presented in milk, the physical environment under the cold storage and the chemical composition of milk (Doyle and Buchanan, 2012).

Milk has high water content, approximately neutral pH and has variously available nutrients which microorganisms can use as carbon and nitrogen sources. The major nutritional components and normal concentrations of milk are 87.3% water, 4.8% lactose, 3.7% fat, 3.2% proteins (casein 2.6% and whey protein 0.6%) and 1% non-protein nitrogenous, minerals and vitamins (D'Aoust et al., 2001). Microorganisms or their enzymes degrade carbohydrates, proteins, and fats which involve the spoilage of milk. The chemical and physiological identification of these microorganisms in milk must be developed for the better detection methods and prevention (Nollet and Toldra, 2010). Today, an important factor is the open dating of

perishable milk to indicate the time to remove milk from the supermarket shelf. Many dairy factories consequently need accelerated investigations to determine the shelf-life. The predictively key shelf life is not the total plate count suddenly after processing since this count does not discriminate between the contaminating bacteria and bacterial survival after pasteurisation. The shelf-life depends on the number of post-pasteurisation contaminants, which multiply rapidly at refrigeration temperatures namely the gram-negative psychrotrophs (especially the pseudomonads). Some microorganisms as low as one or two bacteria per litre are meaningful to the manufactural product with an extended shelf life; for this reason, a storage temperature of 5-7 °C is recommended. (Robinson, 2002).

Detection methods of microorganisms are important in milk spoilage. Standard plate count method is traditionally used to investigate psychrotrophic bacteria in milk but requires plates to be incubated at 7 °C for 7-10 days. This method is time-consuming and labour-intensive. The molecular biological, biochemical and immunological techniques have been applied for rapid and specific detection of microorganisms to overcome the limitations. Fast and straightforward culture-independent method is required for detection of psychrotrophic bacteria in milk, which is considered to be the most important spoilage microbiota. Molecular-based method to analyse DNA or RNA without microbial enrichment has allowed more precise descriptions of microbial dynamics (Nollet and Toldra, 2010). Real-time PCR is one of the most powerful techniques for detection of the spoilage microorganisms. This technique is versatile, rapid and sensitive. (Martínez et al., 2011)

The dairy industry must be able to determine numerously viable bacteria and obtain results within 2-3 h. Fourier transform infrared (FT-IR) spectroscopy is a

metabolic fingerprinting technique which can be used to reduce the detection time. It can detect a significant amount of the biochemical fingerprint data quickly with rather a low cost. Achieving quantitative estimations of many biochemical components is possible. The utilisation of an attenuated total reflection (ATR) accessory of FT-IR spectroscopy keeps a mean to investigate the widely principal components with little or no sample preparation (Li-Chan et al., 2010). Recently, this technique showed the partial least square regression (PLSR) analysis with 100% of useful classification, a good correlation ($R^2 = 0.99$) and very small root mean squares error of validation in the bacterial prediction on chicken spoilage (Sahar and Dufour, 2014).

FT-IR spectroscopy has been irradiated with the infrared region (mostly in the mid-IR range; 4000-400 cm^{-1}), used for detecting the characteristic, absorbance frequencies and primary molecular vibrations of all molecules. When mid-IR radiation passes through a sample, specific wavelengths are absorbed causing the functional groups in the sample vibrate and go to the excited state with stretching and bending. The absorption of bond vibrational energy change in the IR region can be detected as spectral peaks. These peaks correlate to the chemical structures of the molecule and then can provide a quantitative concentration of bacteria (Davis and Mauer, 2010).

The advantage of this technique using the relatively multivariate statistics by the established statistical model such as PLSR analysis gives the spoilage bacterial detection results within 5 min including the time necessary for collecting spectrum, converting data and analysis. This advantage makes FT-IR spectroscopy as a highly attractive technique for routine testing and process control in the dairy industries since

it is necessary to predict the spoilage milk and the formation of metabolic byproducts (Li-Chan et al., 2010).

The objectives of this study were to investigate the feasibility of FT-IR spectroscopy for detection of the bacterial load in milk and to quantify the specific spoilage microorganisms in the pasteurised cow's milk from shelves in the supermarkets.

1.2 Research objective

The objectives of this study were to investigate the feasibility on evaluation for rapid detection of bacterial spoilage detection in milk by a detected spectrum of compositional change of milk using Fourier Transform Infrared Spectroscopy (FT-IR) spectrometer, focal plane array (FPA) detector.

1.3 Research hypothesis

FT-IR microspectroscopy combined with Focal-Plane-Array (FPA detector) could apply to quantify the bacterial levels in milk and could be used to predict the bacterial amount which effects the bacterial spoilage in milk.

CHAPTER II

LITERATURE REVIEWS

2.1 Milk as growth media

Milk is fluid secreted by the female mammals for their infants' nutrition. The Codex Alimentarius Commission (CAC) defines milk as the normal mammary secretion of mammals acquired from one or more milkings without either addition to it or extraction from it, intended for consumption as liquid milk or further processing (Standard, 1999). Cow's milk is many favourites for the world's population and is the vast majority of processed milk for human consumption. Rapid milk spoilage can occur from natural enzymes and contaminating microorganisms. The major component of milk is water, the remaining consists of fat, lactose, protein (casein and whey proteins), small minerals, specific blood proteins, enzymes and low intermediate substances of mammary synthesis. The composition of milk is varied because of dairy breeds, original genetic, results of environmental and physiological factors (Robinson, 2005).

2.1.1 Lipids

The cow's milk has the major lipid component as triglyceride about 98% of milk fat. The other 2% of milk fat consists of diglycerides, monoglycerides, cholesterol, phospholipids, free fatty acids, cerebrosides, and gangliosides. Milk fat is essential to the flavour of dairy products and also off flavours that

may develop in milk. More than 95% of the fat in milk exists in the globule forms, called a milk fat globule membrane (MFGM). MFGM derives from specific regions of the apical plasma membrane of the secretory cells. The membrane represents a natural emulsifying agent, allowing the fat to disperse throughout the aqueous phase of milk, preventing some flocculation and coalescence. (Robinson, 2005).

2.1.2 Proteins

The cow's milk contains about 3.5% of protein. Normal bovine milk fractionates into two groups. About 80% of the total milk proteins at pH 4.6, 20 ℃ precipitates out called casein. The remaining proteins are soluble which is called whey proteins or serum proteins.

2.1.2.1 Casein and Casein Micelles

Fractionated caseins are divided into four distinct proteins: α_{s1}-, α_{s2}-, β-, and κ-caseins. There are also several derived caseins, resulting from the action of indigenous milk proteinases, especially plasmin. These are usually referred to as γ-caseins. The caseins are all phosphoproteins with the phosphate groups which are esterified to the serine residues in the protein chains. The phosphate groups bind with a significant amount of calcium and are critical to the structure of casein micelles (Robinson, 2002).

2.1.2.2 Whey proteins

The major whey protein fractions are β-lactoglobulin, bovine serum albumin (BSA), α-lactalbumin and immunoglobulins. β- lactoglobulin is the most abundant whey protein and represents about 50% of the total whey protein in cow's milk. β-lactoglobulin contains two internal disulphide bonds and a single free thiol group which is the great important cause for changing milk during heating.

α-Lactalbumin which is about 20% of the whey proteins, contains four interchain disulphide bonds. α-lactalbumin binds two atoms of calcium very vigorously and is susceptible to denature when these atoms are removed.

Serum albumin in cow's milk is identical to the serum albumin found in the blood and represents about 5% of the total whey proteins. The protein is synthesised in the liver and compose in milk through the secretory cells that have one free thiol and 17 disulphide linkages, which hold the protein in a multiloop structure.

Immunoglobulins are antibodies that animals synthesise in response to stimulated foreign macromolecular antigens. Four types of immunoglobulins in cow's milk are IgM, IgA, IgE and IgG.

Other proteins are found in small quantities in whey including β-microglobulin, lactoferrin, transferrin, proteose peptones and a group of acyl glycoproteins (Robinson, 2002).

2.1.2.3 Milk salts

Milk salts mainly consist of chloride, phosphates, citrates, sulphates, and bicarbonates of sodium, potassium, calcium and magnesium. Milk salts are soluble and present as ions dissolved in milk whey. Calcium and phosphate are less soluble at the proper pH of milk and partly are insoluble which are closely associated with the casein micelles. Calcium phosphate between the dissolved and colloidal states significantly influences the properties of milk. (Robinson, 2002).

2.1.2.4 Lactose

Lactose, the major carbohydrate in milk is found in cow's milk at 4.8%. This level of lactose does not make milk unduly sweet because lactose is less sweet than sucrose and also less sweet than an equimolar mixture of galactose and

glucose. Lactose makes a major contribution to the colligative properties of milk (osmotic pressure, freezing point depression, boiling point elevation) and exists in both α- and β-lactose forms. Compare with many other sugars; lactose is relatively less soluble in water; its solubility at 25 ℃ is only 17.8 g/100 g solution. Lactose crystals can cause some manufacturing problems because of gritty in texture and relatively low solubility. Crystallization of lactose is also responsible for caking and lumping of dried milk during storage. Lactose, like other reducing sugars, reacts with free amino groups of proteins and gives products which are brown (Robinson, 2002).

2.2 Heat treatment conditions

Pasteurized milk does not become sterile, but the number of microbes is reduced rapidly. Louis Pasteur first described the principle of pasteurisation in 1860, but in modern milk processing, pasteurisation is a set of precisely controlled steps guaranteed on an industrial scale (Roller, 2012). Pasteurization is the most critical safety step for raw milk. Although all attempts to minimise the contaminations are made at the farm level, milk still contains the foodborne pathogens. Vegetative cells of foodborne pathogens are sensitive to the pasteurisation process. During the process, spoilage microorganisms and undesirable enzymes (lipases and protease) are also reduced. Therefore pasteurisation both ensures safety and extend shelf-life with minimal changes to flavour and nutritional quality of the product. Three different heat treatment conditions are usually applied (Motarjemi et al., 2014).

2.2.1 Low temperature–long time (LTLT)

Sixty-three-degree Celsius for at least 30 minutes is the low temperature–long time (LTLT) method used for batch pasteurisation. In this process,

the milk is stirred regularly to guarantee that all particles receive adequate heat treatment.

2.2.2 High temperature–short time (HTST)

Seventy-one point seven-degree Celsius for at least 15 seconds is the high temperature–short time (HTST) pasteurisation method applied in heat exchangers that process milk continuously. The method provides higher energy efficiency.

2.2.3 Ultra-high temperature (UHT)

One hundred and thirty-five-degree Celsius for 1 second is the ultra-high temperature (UHT) process combined with aseptic packaging, the unopened product is shelf-stable and can be kept unrefrigerated. Table 1 indicates the three principal methods used for milk pasteurisation.

Table 2.1 Time-temperature combinations used in the pasteurisation of milk (Roller, 2012).

Treatment	Holding temperature	Minimal time at holding temperature	Chill storage needed?	Typical shelf life
LTLT	63 °C	30 minutes	Yes	2 weeks
HTST	72 °C	15-20 seconds	Yes	2-3 weeks
UHT	135-150 °C	1-3 seconds	No	2-3 months (6-9 months if combined with sterile handling and packing)

2.3 Microbial spoilage of milk

Shelf-life is the length of time kept under reasonable storage conditions that make the dairy products maintain the acceptable characteristics, for example, odour flavour, texture, and physical appearance. The beginning quality and shelf-life stability of pasteurised milk are influenced as follow; 1) the microbial cells and heat-resistant enzymes in raw milk and other ingredients used in manufacturing (Barbano et al., 2006). The 2) activity of essential and microbial enzymes associated with the raw milk. 3) the types, initial numbers and capability growth of microorganisms surviving after the heat treatment processes. 4) the incidence, types, and growth of microorganisms recontaminated into the product after the heat treatment. In general, apparent sensory defects correlated to microbial growth do not occur until bacterial populations exceed 10^6 -10^7cfu/ml (Robinson, 2002). Spoilage of dairy milk is manifested as off flavours, odour and change in texture and appearance. Some defects of milk caused by microorganisms are listed in Table 2.2.

2.3.1 Psychrotrophic bacteria

Spoilage bacteria can be present in the freshly pasteurised milk at a too low level to be detectable (Eneroth et al., 1998), it may be below to the detection limits by conventional enumeration methods which are <10 cfu/ml. However, if the milk is stored at the refrigerated temperature before analysis, they will reproduce and multiply at the refrigerated temperature and can be identified. Cousin (1982) explained some psychrotrophic bacterial milk contaminants with a generation time of less than 6h at a temperature less than 7 °C, if this bacterium reliably replicates every 6h, the bacterial numbers can exceed 10 million after seven days. Therefore, low level of the pasteurised milk contamination can influence the product shelf-life. Sørhaug

and Stepaniak (1997) described the spoilage without bacterial growth from the accumulation and action of extracellular proteolytic and lipolytic enzymes which produced by psychrotrophic bacteria from dairy products (especially by *Pseudomonas* spp.). Psychrotrophic bacteria commonly found in raw milk and were inactivated by pasteurisation (D'Aoust et al., 2001).

Table 2.2 Some defects of fluid milk that result from microbial growth (D'Aoust et al., 2001).

Defect	Associated microorganisms	Type of enzyme	Metabolic product
Bitter flavour	Psychrotrophic bacteria, *Bacillus* spp.	Protease, Peptidase	Bitter peptides
Rancid flavour	Psychrotrophic bacteria	Lipase	Free fatty acids
Fruity flavour	Psychrotrophic bacteria	Esterase	Ethyl esters
Coagulation	*Bacillus* spp.	Protease	Casein destabilization
Sour flavour	Lactic acid bacteria	Glycolytic enzyme	Lactic, acetic acids
Malty flavour	Lactic acid bacteria	Oxidase	3-Methyl butanal
Ropy texture	Lactic acid bacteria	Polymerase	Exopolysaccharides

2.3.2 Spore-forming bacteria

The psychrotolerant endospore-forming bacteria can occur in HTST pasteurised milk and be a key hurdle to extend the product shelf-life to 14 days. Heat-resistant psychrotrophs consist of members from the genera *Bacillus, Clostridium*,

Arthrobacter, *Microbacterium*, *Streptococcus* and *Corynebacterium*. However, the predominant microorganisms are *Bacillus* species which introduce into milk supplies from water, udder and teat surfaces or soil and milestone deposits on-farm bulk tanks, pumps, pipelines, gaskets, and processing equipment. In the spore state, these microorganisms easily survive during the typical range of pasteurisation conditions and germinate to be vegetative cells producing degradative enzymes, for example, proteinases, lipases, and phospholipases. Enzymatic activity results in the development of unpleasant flavour and quality defects in dairy products (Meer et al., 1991). The seasonal occurrence of *Bacillus* and *Clostridium* spp. in raw milk, which are collected in the winter, are higher levels than in the summer. Because in the winter, cows lie on spore-contaminated bedding materials and are likely to consume spore-afflicted silage (D'Aoust et al., 2001).

2.3.3 Pathogenic microorganism

From 1998 to 2005, data in the USA indicated 39 outbreaks causing 831 cases of 66 hospitalisations and one death and these were related to the consumption of raw milk. (Motarjemi et al., 2014). The most regular method for destroying pathogenic organisms and for eliminating spoilage organisms in United State dairy products is through pasteurisation by the HTST method (D'Aoust et al., 2001). More recently, concerns were increased by arguably research results showing occasional survive of *Mycobacterium avium* subsp. *paratuberculosis* in pasteurised milk. The controversy as regards this bacterium can survive in pasteurised milk, but now it is not a major issue since it may reappear on other opportunities with other emerging pathogens (Condron et al., 2015).

2.3.4 Standards for microorganisms in ready-to-drink milk

The Minister of Public Health, Thailand at this moment issues the notification of the Ministry of Public Health No. 350 (2013) Re: Cow's Milk and No.364 (2013) Re: Standards for Pathogenic Microorganisms in Food. Ready-to-drink milk proceeded through the heat treatment process by pasteurisation shall be of qualities or standards as follows:

Table 2.3 Criteria of Pathogenic microorganisms in pasteurised milk.

Type of microorganism	**Criteria**
Escherichia coli	Must not be detected in 0.1 ml
Bacterial count	Must be detected not more than 10,000 in 1 ml at manufacturing factory and not more than 50,000 at all-time after the departure from manufacturing to the expiry date on the label.
Coliform bacteria	Must be detected not more than 100 in 1 ml of pasteurised cow's milk at the manufacturing factory.
Salmonella spp.	Must not be detected in 25 ml
Staphylococcus aureus	Must not be detected in 0.1 ml
Bacillus cereus	Must not more than 100 cfu/ml
Listeria monocytogenes	Must not be detected in 25 ml

2.4 Detection methods of spoilage microorganism

Some different techniques have been explored and utilised over the years for the detection and enumeration of microbiological spoilage in milk. These include ATP bioluminescence, phase-contrast microscopy, immunofluorescence assays, polymerase chain reaction, electronic nose techniques, each with positive and negative aspects in regards to their application and outcome.

2.4.1 Adenosine triphosphate (ATP) bioluminescence

A bioluminescence technique measures the ATP content in food as an indirect measurement of microbial load. As only the viable cells retain ATP, the total of ATP is regarded as directly related to the microbial load in the food. Using the luciferin-luciferase system in the presence of Mg^{2+}, the amount of ATP in the lysed cells in a sample is determined. This method can detect as low as 10^2 viable bacterial cells of food. The method is very rapid and various automated systems are now commercially available, for example, bioluminescence method to determine isolated bacterial ATP from meat juice. Immunoparticles are used to specifically capture and isolated the bacteria before bacterial ATP release in the presence of luciferin-luciferase system to provide the optical (luminescence) signal (Singh et al., 2016). Shinozaki and Harada detected bacteria in green tea and evaluated *Enterobacter cloacae*, *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* using bioluminescence assay. The results showed that the microbial detection was 15 cfu in 500 ml of bottled green tea after the 8 h incubation at 35 °C and an assay time of 1 h (Shinozaki and Harada, 2014).

2.4.2 Phase-contrast microscopy

Small and easy-to-use phase-contrast microscopes are available to rapidly identify microbial types (morphology, motility, spore, and cell arrangement) which present in food. However, the population has to reach a relatively high level (10^{5-6}/ml) before it can be viewed under a phase-contrast microscope. Also, food particles can interfere with the identification. With practice, it can be a rapid and easy method to get initial ideas about the predominant microbial types. It is also possible to do fast and direct enumeration of cells by a suitable counting device (e.g., Petroff Hauser counter). The results can be explained in several ways. If the desired level is set (specification level), such as a spoilage-detection level, one can interpret the result as less than the level (desirable), very close to the level (should be used immediately), or above the level. For a nonliquid food, a known amount of food can be suspended in sterile water in a 1:1 dilution, mixed well, and one to two drops of supernatant fluid can be used on a microscopic slide or a counter for viewing or counting. Even phase-contrast microscopy provides immediate identification, but it is not quantitative the microbes in food (Robertson, 2000). The application of various phase-contrast microscope has been used to identify microorganisms from spoiled foods such as *Sporolactobacillus nakayamae* spores from spoiled foods are confirmed by phase-contrast microscopy (Bozkurt et al., 2016). In addition, Wang et al. (2015) determined effects of berberine on spore germination and outgrowth of *Bacillus subtilis* that observed by phase contrast microscopy.

2.4.3 Immunofluorescence assays

In principle, an immunofluorescence (IF) assay similar to an Enzyme-linked immunosorbent assay (ELISA), it is one of the most used immunoassays up to

now (Priyanka et al., 2016). In IF, a fluorescently labelled antibody that emits fluorescence after forming a complex with an antigen on a glass slide in a 96-well microtiter plate is used, which is detected by a fluorescence microscope, a digital camera, or a spectrofluorometer. The fluorescent markers are used rhodamine B, fluorescein isocyanate and fluorescein isothiocyanate (FITC). The fluorescent antibody method can be performed by using two basic methods: The direct method uses binding of antigens with a fluorescent-labelled specific antibody. In the indirect method, the primary antibody is not coupled with the fluorescence, but an alternative species-specific secondary antibody is labelled with fluorescence. In the indirect method, the labelled antibody detects the presence of the primary antibody and antigen complex. The employ of the indirect method excludes the requirement to prepare a fluorescent-labelled antibody for each microorganism of interest. One such research was developed for rapid and sensitive detection of *Escherichia coli* O157:H7. This bacteria is a primary concern for the food industry, fast and sensitive detection of this pathogen is essential to ensure food safety. The immunofluorescence assay with the immunomagnetic beads consists of two reaction steps: Immunomagnetic separation and fluorescence immunoassay with tyramide-signal amplification (TySA). This immunoassay is able to detect *E. coli* O157:H7 in pure culture with a detection limit of 50 cfu/ml in less than 3 h without an enrichment culture (Aydin et al., 2014).

2.4.4 Real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR), a fast and inexpensive quantitative method to detect the presence of targeted DNA-segments of bacteria in foods. Because of its prompt and most sensitive and specific technique for

species identification, especially since RT-PCR has performed when target concentrations are deficient, limit of detection at 95% confidence is three molecules (Forootan et al., 2017). Conventional PCR is a cyclic process involving denaturation, annealing, and extension steps that double the target sequences after each cycle. Regarding RT-PCR, which is an automated process, no post-PCR processing is required to analyse the amplification process, as it monitors the increasing copy number of amplicon in real time after each cycle (Salihah et al., 2016). Several RT-PCR assays have been developed for the quantitative detection of food spoilage microorganisms, such as *Alicyclobacillus* spp. in fruit juices (Cai et al., 2015), Lactic acid bacteria in wine (Kántor, 2014) and *Bacillus cereus* group spores in food (Martínez-Blanch, 2010). So the food industry can use for screening and quantifying food spoilage microorganisms to improve the safety and quality of its products.

2.4.5 Electronic nose techniques

Electronic noses (e-noses) are a tool which mimics the sense of smell. These devices are typically an array of sensors use to determine and distinguish odours precisely in complex samples and at low cost. E-noses are very useful for diverse applications in the food, cosmetic and pharmaceutical industry. (Peris and Escuder-Gilabert, 2009). Currently, the researcher's attention is mostly focused on reducing the time of a single analysis, increasing the sensitivity of the measuring set up, miniaturisation and validation of the employed methods. The term of the e-nose may bring to mind a tool with capabilities similar to the human olfactory apparatus, where in fact the main appearance in which e-nose resembles to its biological counterpart to its function. The sensor's response signal is subsequently transmitted to a computer, where pattern recognition is performed (Wojnowski et al.,

2017). One example, the application of a fast electronic nose system (Cyranose 320) for detecting the total viable counts (TVC) from fresh and frozen beef samples were determined using the standard plate count method and by the application of the e-noses. The results revealed that the Cyranose 320 could detect the TVC in various beef and sausage samples and quantify the volatile organic compounds produced at concentrations from 50 ppb to > 350 ppb. From this study, one can conclude that the electronic nose system is a rapid way of detecting volatile organic compounds produced by bacteria that contaminate in beef (Abdallah, 2013).

2.4.6 MALDI-TOF mass spectroscopy

Matrix-assisted laser desorption/ionization-time-of-flight massspectroscopy (MALDI-TOF-MS) is a proven technique in the field of protein and peptide identification and quantification, may be a valuable alternative approach for the rapid assessment of microbial spoilage (Nicolaou et al., 2012). MALDI-TOF-MS has been already applied to determine *L. monocytogenes* in Australian dairy products (Jadhav et al., 2015). *Escherichia coli* from a dairy cattle environment are also identified in Brazil dairy products using MALDI-TOF-MS fingerprinting (Rodrigues et al., 2017). It has also been used to develop using the vitreous fluid of fish eyes may represent a valuable alternative to currently available methods. The fish eye is a closed environment, and the vitreous fluid is usually not exposed to bacterial spoilage and external influences. The sampling is non-destructive, fast and reliable and the results can be interpreted without exact knowledge of MALDI-TOF MS if the suitable software is used (Ulrich et al., 2017).

2.5 Fourier transforms infrared spectrometry

2.5.1 Infrared spectroscopy

Infrared spectroscopy has been one of the most widely studied sensor technologies for the prediction of milk quality. (Nicolaou and Goodacre, 2008). Figure 2.1 shows the wavelength and wave number ranges of IR regions concerning mid-IR given in the following, but near-IR and far-IR are not the focus here.

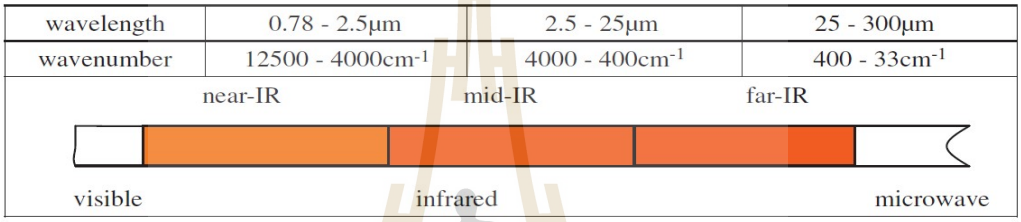

Figure 2.1 The spectrum ranges of IR (Ratner, 2013).

Mid-Infrared spectroscopy or Fourier transform infrared spectroscopy (FT-IR) is based on the mid radiation in the 4000-400 cm^{-1} region of the electromagnetic spectrum providing a rapid characterisation of quality. Measuring principle for the analysis of milk, the success of the FT-IR has been effectively commercialized with dairy products such as the MilkoScan™ FT 120 which operates this principle in agreement with AOAC standards (Nollet and Toldra, 2010). Mid-IR represents the absorption spectrum of all the chemical bonds, such as O–H, N–H, C–O, C–H and others. The spectrum is sensitive to the physical and chemical states of individual components in a sample between 4000 and 400 cm^{-1}. The most general use of mid-IR is to determine the chemical functional groups in the sample. Different functional groups demonstrate the different absorb characteristic frequencies of infrared radiation in the mid-IR region (Figure 2.2).

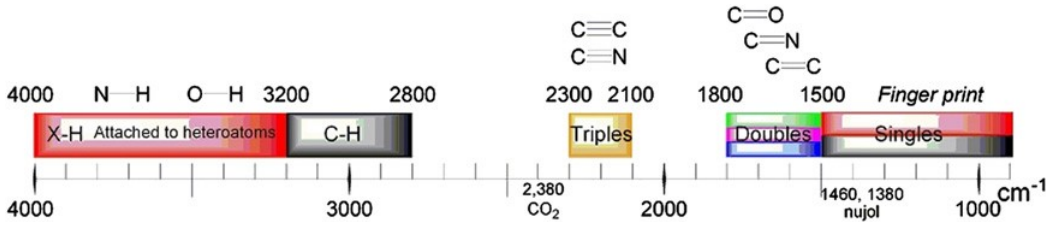

Figure 2.2 The absorptions of the functional groups in Mid-IR region (Zhang, 2012).

Based on a FT-IR spectrometer in Figure 2.3, the experimental system composes of mainly four parts: a reference blackbody, a heating furnace for a sample, an optical system for guiding the light from the black body or the sample and the FT-IR spectrometer.

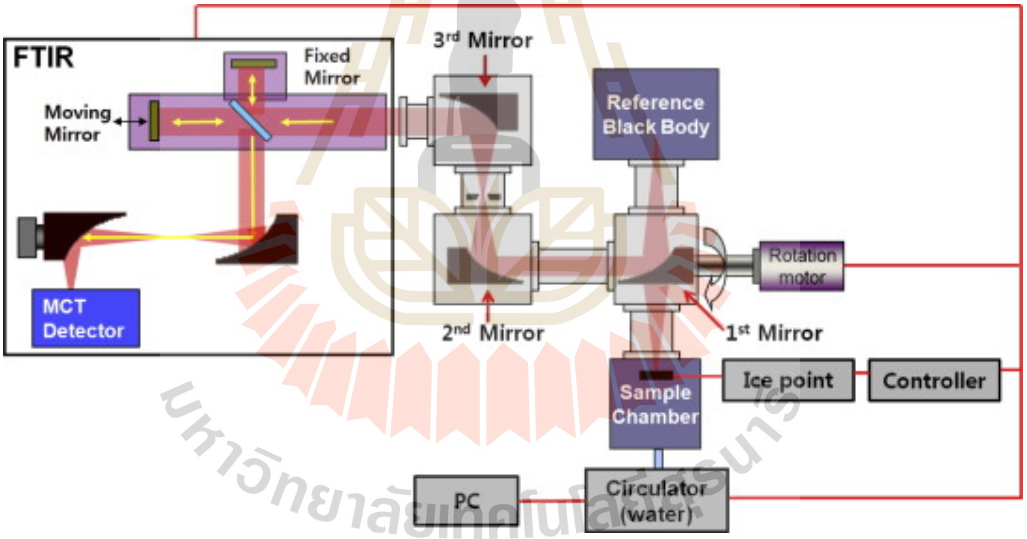

Figure 2.3 Schematic diagram of an instrument for measuring IR properties using a FT-IR spectrometer (Jeon et al., 2013).

2.5.1.1 FT-IR hardware

FT-IR equipment and instrumentation have changed significantly over the years. Currently, various types and arrangements of instruments are available and have specific applications. Thus, it is essential to comprehend the basic construction of a FT-IR. A figure of a FT-IR spectrometer is shown in Figure 2.4. The major components belong to the IR source, a beam splitter, detector and reference laser. The set-up includes reflecting mirrors at many points to direct the path of IR light. The light from the source moves through the aperture wheel and hits a mirror that directs the light onto the beam splitter. Mirrors then direct the recombined light from the interferometer into the sample compartment, finally detected by the detector (Sun, 2009).

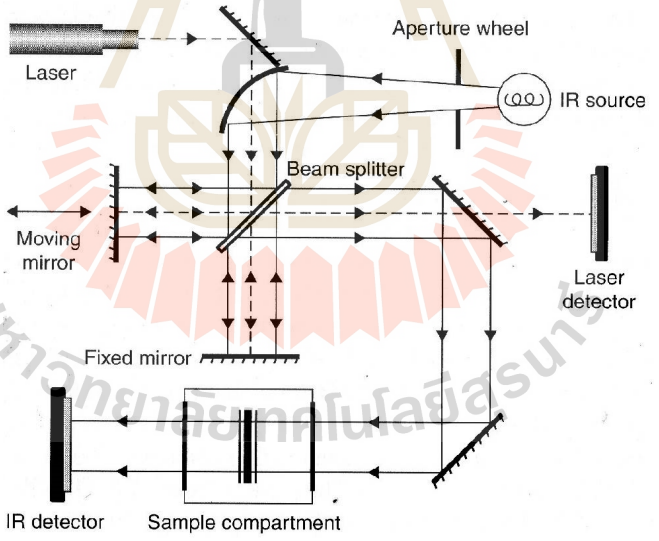

Figure 2.4 Optical layout of a typical FTIR spectroscopy (Sun, 2009)

2.5.1.2 The source

The function of the IR source in the spectrometer is to emit IR radiation and generate heat due to the resistance of the source to conduction of

current. The resistance heats up the source to above 800°C making IR source to emit IR radiation. Since the high operating temperatures occur, a cooling system is needed. Based on the cooling system, FT-IR sources are either two types. (1) Water-cooled sources which are called globar sources, made of silicon carbide, providing high throughput, require a constant flow of water for cooling and are expensive. (2) Air-cooled sources which made of ceramic or nichrome wire and inexpensive. Normally, sources are fixed in front of a different mirror to capture leaving light and direct it towards the samples (Sun, 2009).

2.5.1.3 Beam splitters

Serving to split and recombine the IR light waves in the interferometer, beam splitters are constructed by sandwiching a coating semi-transparent material between IR-transparent substrates. Three types of substrates are used in FT-IR instruments: quartz, calcium fluoride (CaF_2) and potassium bromide (KBr). The coatings alter, depend on the manufacturer. Potassium bromide substrate with germanium (Ge) coating splitter is the most widely used for Fourier transform measurements. Also, the Br beam splitter works very well in the MIR region (4000-400cm^{-1}) which is hard but hygroscopic. Thus, many of the FT-IR spectrometers require purging with moisture-free air or nitrogen (Sun, 2009).

2.5.1.4 Interferometer

One of the most important components is the moving mirror, being essential to control the position correctly to obtain an accurate measurement of spectra. Two types of mechanisms are generally used to move the mirror are air bearings and mechanical bearings. In air bearings, the moving mirror is airy. This types are frictionless but expensive and require a constant source of clean and dry air

and be disturbed by vibrations. The mechanical bearing system is inexpensive, generally uses a ball-bearing (Sun, 2009).

2.5.1.5 Detectors

The function of the detector is to transduce the light intensity acquire by it to an electrical signal, the most commonly used detector in FT-IR instruments is deuterated triglycine sulphate (DTGS) detector and the mercury cadmium telluride (MCT) detector. In a DTGS detector, a change in the intensity of IR radiation attacks the detector causing a proportional change in temperature, which in turn will cause a change in the dielectric constant of deuterated triglycine and the capacitance can be measured as the detector response in voltage. Being very simple and inexpensive, but DTGS detector has a relatively slow response and low sensitivity. The MCT detector is a semiconductor, the electron conducting bands generate an electrical current proportional to the IR intensity, present in the absorbed IR light and move from valence band to conduction band. MCT detectors are more sensitive and faster than DTGS detectors and hence providing a spectrum with higher SNR. Disadvantages of MCT detector is a narrow bandwidth based on the composition and saturates very easily. MCT detector requires cooling liquid nitrogen. Improper cooling will result in a signal, and MCT detectors cost more than DTGS detectors. The electrical signal produced by the detector is converted to voltage, amplified, processed and converted from analog to digital using digital converters. The digitised signals are then Fourier transformed (Sun, 2009).

2.5.1.6 Laser

Modern FT-IR instruments are equipped with a red He-Ne laser, which gives light at exactly 15798.637 cm^{-1}. The laser serves two purposes.

First, since its wavenumber is known precisely, it acts as an internal wavenumber standard based on which other wavenumbers are measured. The wavenumber reproducibility of most FT-IR spectrometers is $\pm$0.01 cm^{-1} or better. Second, it is used to determine the position of moving mirror. The detector response is also used for checking and aligning the optical interferometer (Sun, 2009).

2.5.2 Fundamentals of FT-IR spectroscopy

Since the beginning of spectroscopy, Mid-IR spectroscopy has a tremendous attraction because of the ability to provide information-rich spectra which enabling to show the structural characterisation of molecules. Advances in FT-IR instrumentation coupled with the development of powerful multivariate data analysis can be the rapid screening and characterising of minor food components at ppb levels. Mid-IR is very robust and reproducible to detect the electromagnetic spectrum in the minimal composition of samples. FT-IR monitors the structural, vibrational and rotation spectroscopy. Stretching of molecules absorb mid-IR energy and exhibit stretching, bending, rocking and scissoring motions at one or more locations in the spectra, depending on several factors including bond configuration, location and producing a chemical profile of the sample. It is well-supplied information which helps in analysing the composition and determining the structure of chemical molecules.

For example, a typical second derivative FT-IR spectrum of *Salmonella* Enteritidis which is shown in Figure 2.5 and Figure 2.6 shows FT-IR spectra of ultra-heated (UHT) whole milk. FT-IR spectra reflect the total biochemical composition of the sample; bands caused major cellular constituents such as water, lipids, polysaccharides, acids and so on. The region from 4000 to 3100 cm^{-1} composers of

the density from O-H and N-H stretching vibrations of hydroxyl groups and amide A of proteins, respectively. Protein bands also are in sight in the regions 1700-1550 cm^{-1} (amide I and amide II) and 1310-1250 cm^{-1} (amide III). The C-H stretching movement of $-CH_3$ and $> CH_2$ functional groups appear between 3100 and 2800 cm^{-1}, the spectral range 1250-800 cm^{-1} compose of signals from phosphodiesters and carbohydrates. From 1200 to 600 cm^{-1} called the "fingerprint region" since this region contains signals being distinct between each sample and highly maintained within each sample (Sun, 2009). Some attributed bands of milk components can be deduced from Table 2.4.

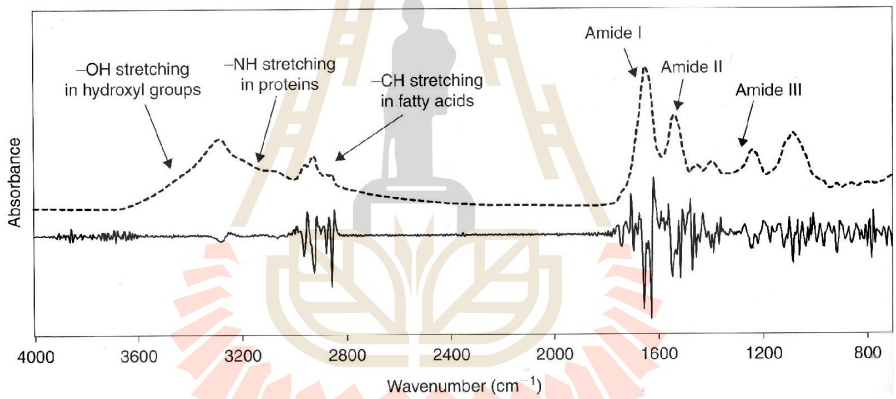

Figure 2.5 Raw (dashed line) and second derivative (solid line) mid-IR (4000-700 cm^{-1}) spectra of *Salmonella* Enteritidis prepared in distilled water and measured on a three-bounce zinc selenide attenuated total reflectance crystal (Sun, 2009).

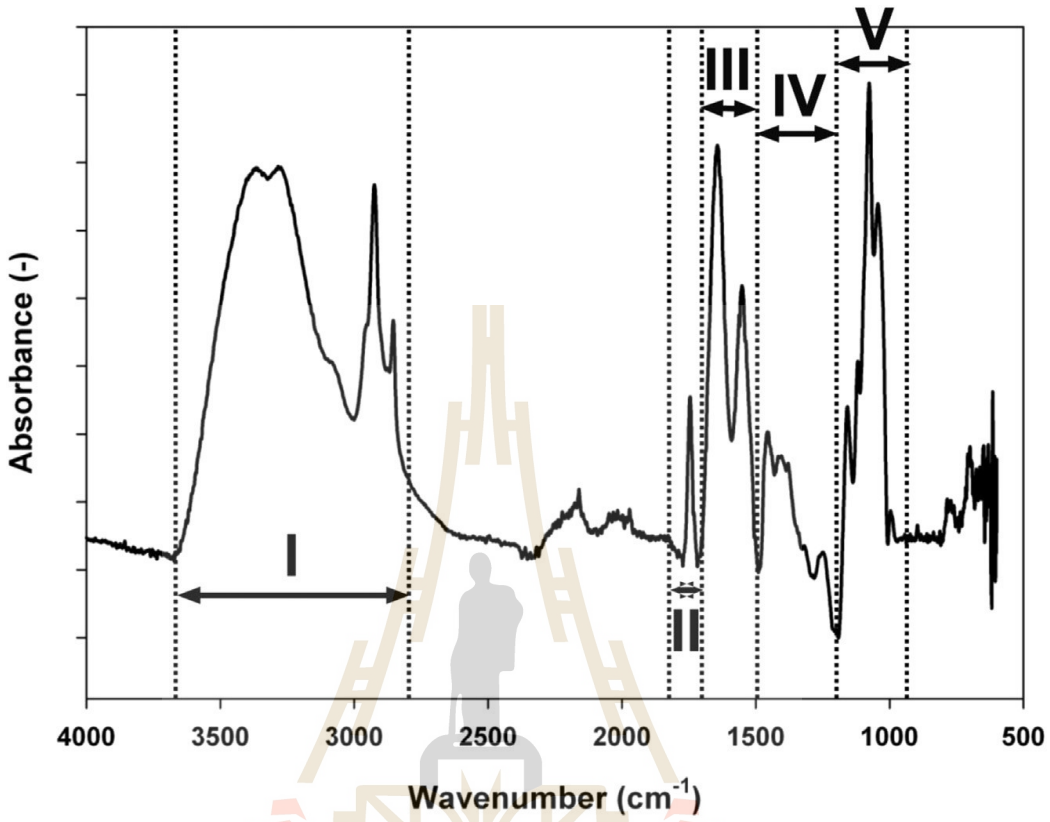

Figure 2.6 FT-IR spectra of ultra-heated (UHT) whole milk on day 0 of storage from 4000-600 cm^{-1}, 3700-2800 cm^{-1} (Region I), 1800-1700 cm^{-1} (Region II), 1700-1500 cm^{-1} (Region III), 1500-1200 cm^{-1} (Region IV) and 1200-900 cm^{-1} (Region V) (Grewal et al., 2017).

Table 2.4 Milk components attributed to different regions of FT-IR spectra (Grewal et al., 2017).

Wave number (cm^{-1})	**Milk component**
3000-2800	Milk lipids
1800-1700	Milk lipids
1700-1600	Milk proteins-amide I
1600-1500	Milk proteins-amide II
1500-1200	Milk proteins-amide III, interactions between different milk components (Fingerprint region)
1200-900	Milk minerals; milk fat, lactose (Fingerprint region)

2.5.2.1 FT-IR microspectroscopy

Since the capabilities of FT-IR by enabling visual and can analyse at the micron level of samples, the IR microscope has widely gained acceptance as a very useful microanalytical tool for identifying and characterising chemical and biological samples. The assembly of an IR microscope in principle is the same as most optical microscopes, IR radiation from the spectrometer directed onto the sample through the series of mirrors and lenses. The light emerges from the sample channel into a detector, the detector in most modern microscopes is an MCT detector. The IR microscope is a costly accessory but has the diversified possible applications by enabling to be microanalysis and increasing the sensitivity and speed of detection. Modern microscopes allow both transmission and reflectance. Applications of FT-IR microspectroscopy evolve in food safety or microbial characterisation.

FT-IR microspectroscopy with its unique advantages has excellent potential as an analytical tool and has recently opened up a new area of research (Sun, 2009).

FT-IR imaging incorporates an interferometer, IR microscope and array detector (Figure 2.7). Data is recorded as the spectral multiplexing in time using the interferometer and simultaneously by a large number of detectors. Focal plane array (FPA) detector eliminates the diffraction limit by sandwiching the sample between an array to extend the wavelength sensitivity, non-microscopy imaging attempts and the use of filter-based imaging (Bhargava, 2012; Osawa et al., 2002).

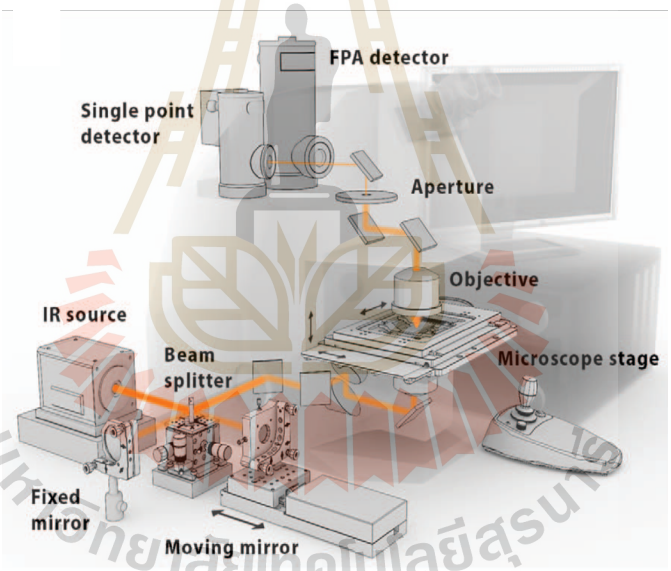

Figure 2.7 Typical layout of a FPA-FT-IR microspectrometer. Instrumentation, only the transmission path is shown here, dual detectors enabling both point mapping and imaging technology and external interferometer as well as a computer to obtain and process data (Bhargava, 2012).

2.5.2.2 Collecting spectra

Most FT-IR instruments are a single beam. The background and the spectrum are collected at separate times. The spectrum of the sample obtained by Fourier transforming interferogram which is called the single-beam spectrum, representing the signal from the sample as well as from the instrument and the environment. The single-beam spectrum is ratioed against the background spectrum obtained without the sample, and the actual spectrum is obtaining from the sample. A schematic diagram of the series of steps are involved in obtaining a spectrum of a sample is shown in Figure 2.8. The signal-to-noise ratio (SNR) is directly proportional to the square root of the number of scans. Therefore, typically multiple scans are added together, during co-addition noise, which is random positive and negative signals, cancels out while the signal intensity remains the same. The good SNR should be used co-adding around 100 scans or more which is in general enough to obtain. It depends on the instrument such as an IR microscope, which is much more sensitive, requiring co-adding more scans to get a good SNR (Sun, 2009).

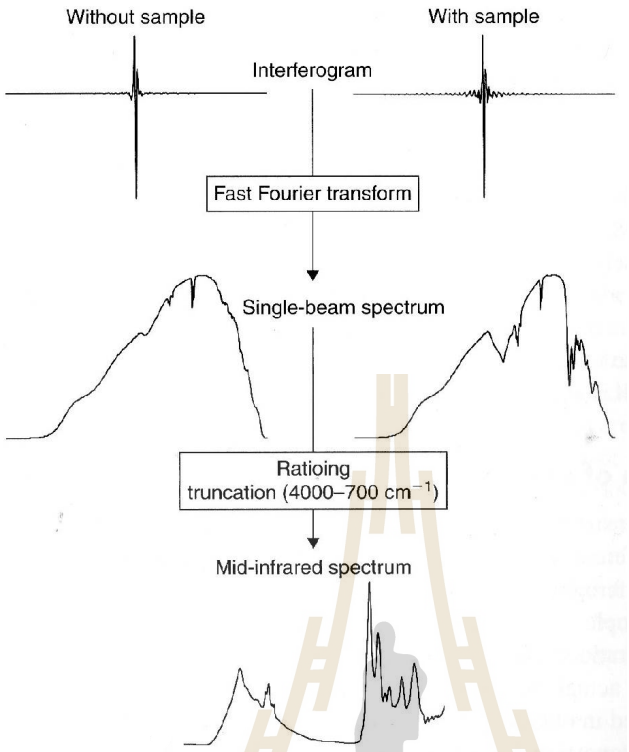

Figure 2.8 An illustration of how a mid-infrared spectrum is obtained from the interferogram (Sun, 2009).

2.5.2.3 Factors affecting FT-IR spectroscopy

Obtaining the highest probable SNR and a good enough resolution for a specific analysis are essential factors when uses FT-IR spectroscopy for analysis. Some factors contribute to a good FT-IR spectrum. The SNR is the ratio of the height of a band in the spectrum to the height of the noise at some point on the baseline of the spectrum. The SNR is directly proportionate to the square root of the number of scans. Therefore, co-adding several scans achieve high SNR. SNR is also directly proportionate to the time spent on measuring a data point. Modern rapid scanning spectrometers are significantly quick thereby increasing the SNR. Spectral resolution is a property of the instrument and distinguishes the position of features in

the spectra. The higher the Optical Path Difference, the greater the spectral resolution. In food analysis using FT-IR, 8 cm^{-1} or 4 cm^{-1} is an excellent resolution to use, the SNR and resolution themselves are directly associated. High-resolution spectra will have more noise due to the divergence of light in the optics, electronic noise, obtaining high-resolution. Another factor to be considered is that co-adding scans and obtaining high-resolution spectra improve the quality of the spectra at the cost of time. Therefore it is essential to collect a few trial spectra before exact experiment for optimising the parameters (Sun, 2009).

2.5.2.4 Application of FT-IR spectroscopy

Mid-IR spectroscopy is the FT-IR spectroscopic method of selection in applications relating to structural characterisation. FT-IR spectroscopy can detect, identify and characterise bacteria verified by (Naumann et al., 1991). Infrared signals of microorganisms are highly specific fingerprint-like patterns used for investigating the identity of microorganisms. The simplicity and versatility of Fourier-transform infrared spectroscopy (FT-IR) make this technique to be rapid differentiation, classification, identification and large-scale screening at the subspecies level.

The IR spectra are the result of bands of fundamental vibrational transitions combined mainly with functional groups. FT-IR can be used for the chemically based discrimination of intact microbial cells and produced complex biochemical fingerprints which being distinct and reproducible for different bacteria. The complex FT-IR spectra reflect the total biochemical composition of the microorganism, their bands caused by major cellular constituents such as lipids, proteins, and polysaccharides. The potential of FT-IR spectroscopy combined with

multivariate analysis can be used for predicting total viable count in UHT milk samples. Nicolaou (2011) developed the partial least squares (PLS) models based on total viable count data and FT-IR spectra of *Staphylococcus aureus.* The correlation of coefficients for the R^2 value for the test set was 0.74 and the R^2 value for the training set was 0.78, with root mean square errors (log) for the prediction of 0.47. Ten PLS factors used for this model were shown in Figure 2.9.

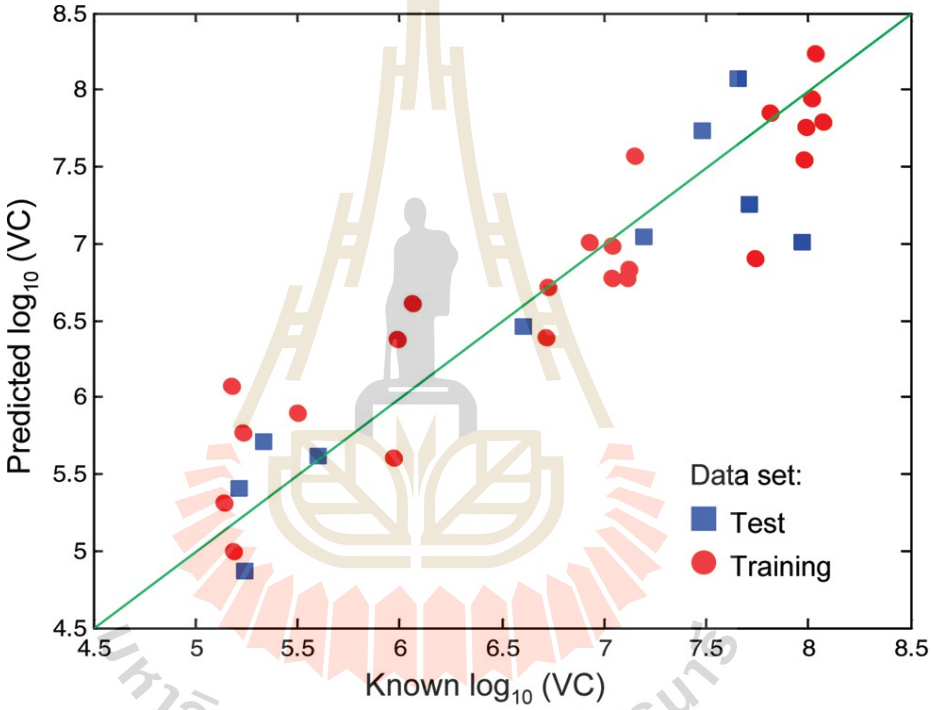

Figure 2.9 Partial least squares regression plot for correlation between FT-IR spectrum and standard plate count of *S. aureus* in mono-culture (Nicolaou et al., 2011).

2.5.2.5 Advantages and disadvantages of FT-IR spectroscopy

The advantages and disadvantages of using FT-IR spectroscopy methods for analysing microorganisms being as follow (Davis and Mauer, 2010).

2.5.2.5.1 Advantages

1) Relatively fast and simple to utilise: Requiring minimum sample preparation.
2) Nondestructive: The bacterial cell remains intact during analysis.
3) Universal method: The instrument and software being readily available and used for routine analysis.
4) Qualitative as well as quantitative analysis: Spectra providing information about bacterial cell composition, quantifying the number of bacteria or amount of functional groups present in a sample.
5) Multiple sample surroundings: Liquid, gas, powder, solid, or film can be tested.
6) Identification and discrimination of bacteria: Bacteria discriminated based on their physiological state, for example, live, dead, injured and treated.
7) Relatively less expensive for bacterial identification compared to several commonly used methods.

2.5.2.5.2 Disadvantages

1) A complete library of spectra for every type of bacteria is recommended to simplify the detection.
2) Require standardisation, precise data collection, and expertise in the chemometric analysis of spectra.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Culture media and materials

Merck's tryptic soy agar; lot number VM611158348, sterile plastic petri dish and materials were obtained from Regional Medical Science Center 9, Nakhon Ratchasima.

3.1.2 Bacterial strains

Bacillus cereus ATCC 11778 and *Pseudomonas aeruginosa* ATCC 15442 were obtained from Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand.

3.1.3 Milk sample

Thai – Denmark's the ultra-heated (UHT) 100% whole milk; lot number EXP. 08/12/16 03:38:56 17 used in this experiment was supported by Dairy Farming Promotion Organization of Thailand (Muaklek, Saraburi).

3.2 Methods

3.2.1 Standard curves of bacterial suspension

Standard curve method was used to select bacterial suspension with a known total viable count. One loopful of culture from nutrient agar slant was inoculated in 100 ml of nutrient broth (NB) and was incubated at 37 °C for 18 h. The

bacterial cells were centrifuged at 4,000 revolutions per minute (rpm) for 10 min. The pellet was washed twice with normal saline (0.85% NaCl) and centrifuged at 4,000 rpm for 5 min. The pellet was resuspended and then was mixed in 10 ml of 0.85% NaCl. The cell suspensions were diluted in the serial dilutions, and spectrophotometer measured absorbance value or optical densities (ODs) with the absorbance range of 0.05-0.25 at a wavelength of 500 nm (Eumkeb et al., 2010; LIU et al., 2000). Total viable counts (TVC) for each absorbance reading were determined in duplicates using spread plate method, and the result was calibrated to produce a standard curve.

3.2.2 Sample preparation

Each colony of *B. cereus* and *P. aeruginosa* were inoculated in each bottle containing NB, then were incubated for 18 h at 37 °C. The ODs of *B. cereus* and *P. aeruginosa* suspensions in 0.85% NaCl were measured and adjusted to approximately 1 x 10^6 cfu/ml by using ODs of bacterial suspension standard curve for each strain. The inoculum of 2 ml of standard suspension of each strain was added to 1 litre sterile flasks containing 498 ml of UHT milk. To give approximately 4 x 10^3 cfu/ml, the first with *B. cereus* ATCC 11778, the second with *P. aeruginosa* ATCC 15442 and the third with co-culture of both *B. cereus* and *P. aeruginosa*. Inoculated milk samples were rotated at 200 rpm, 37 ℃. The total incubation period was 24 h and during this period, sampling was taken at 0, 60, 120, 150, 180, 210, 240, 300, 360, 420, 480, 720, 960 and 1,440 min from all flasks (Nicolaou et al., 2011). Two aliquots were obtained at each sampling point from each flask, one aliquot was used to determine the total viable counts (TVC) using conventionally microbiological plating methods, while another aliquot was separated to be 3 eppendorfs x 1 ml and

preserved at -70 °C collecting for FT-IR microspectroscopy analysis (Nicolaou et al., 2011).

3.2.3 Instrumentation

3.2.3.1 Rotational incubator, deep freezer,-70 °C, weighing, pH-meter, autoclave and hot air oven at Center for Scientific and Technological Equipment (CSTE), Suranaree University of Technology, Nakhon Ratchasima were used to collect, preserve the milk samples and to enumerate the spoilage microorganisms.

3.2.3.2 Bruker IR spectrometer (tensor 27) coupled to an IR microscope (Hyperion 3000) at Synchrotron Light Research Institute (Public Organization) was used for collecting FT-IR spectra.

3.2.4 Total viable counts (TVC)

Total viable counts were measured according to Nicolaou et al. (2011). One ml sample was mixed in 9 ml of phosphate-buffer and was vortexed for 1 min. Spread plate technique was duplicated for each dilution and incubated promptly for 20 h at 37 °C. The plate colonies were counted, and TVC per ml was calculated by multiplying by the inverse of the dilution factor.

3.2.5 Statistical analysis

All cfu/ml were converted to log 10 before statistical analysis and then all statistical analyses were conducted using SPSS software (Statistics Package for the Social Sciences, version 18). Statistical analysis of microbial data was expressed as means ± standard error of means (SEM) and was determined by one way ANOVA followed by Fisher-Hayter test. The results with $p<0.05$ were considered as statistically significant differences. All data from principle component analysis (PCA) and partial least squares (PLS) were created as graphs by SigmaPlot, version 12.0.

3.2.6 FT-IR microspectroscopy, Focal Plane Array (FPA) detector

3.2.6.1 Sample preparation

The previously frozen samples at -70 °C were slowly defrosted in ice, one sample at a time, and was vortexed for 1 min. One microliter was taken from the sample and then air-dried on the Mirr IR low-E glass microscope slides (Kevey slide) which were used for FT-IR microspectroscopy analysis (Osawa et al., 2002). The slides were desiccated under vacuum for several hours and stored in a desiccator to form suitable films before analysis. The spectra were collected by using FT-IR microspectroscopy, FPA detector regarding absorbance obtained by rationing the single beam spectrum against the slide background (Santos et al., 2013).

3.2.6.2 Infrared spectroscopy measurements

FT-IR spectra were acquired with a Bruker Tensor 27 spectrometer (Globar source) coupled with a Bruker Hyperion 3000 microscope (Bruker Optics Inc., Ettlin-Gen, Germany). The microscope was equipped with 64 x 64 element MCT, FPA detector, which allowed simultaneous acquisition of spectral data with a 15x objective. FT-IR samples were recorded in reflection mode, 4 cm^{-1} spectral resolution, 64 scans. Each of the images used to construct 8 x 8 binning FT-IR image mosaic. Absorbance spectra were acquired in the spectral range of 4000–700 cm^{-1}. OPUS 7.5 software (Bruker Optics Ltd., Ettlingen, Germany) was used to obtain FTIR spectral data and control instrument system. The spectra of samples were identified by Principal Component Analysis (PCA) using variability of the Unscrambler X 10.1 software (CAMO Software AS, Oslo, Norway). The spectra were processed using the second derivative and vector normalised by Savitzky-Golay method (9 smoothing points) and then normalised with Extended Multiplicative

Signal Correction (EMSC) in the spectral regions from 3000 to 2800 cm^{-1} and 1800-900 cm^{-1}. Score plots (2 dimensions) and loading plots were used to represent the different classes of data and relations among variables of the data set, respectively.

3.2.6.3 Pre-processing of data

FPA-FT-IR spectra were inspected and checked for outliers using principal components analysis and were investigated the relationship between the FT-IR spectra and the total viable counts using the multivariate statistical methods which were principle component analysis (PCA) and partial least squares (PLS) (Nicolaou and Goodacre, 2008).

3.2.6.4 Partial least squares (PLS)

Partial least squares (PLS) was employed for the four different spoilage experiments to predict bacterial quantification of the total viable count from the FT-IR spectra. Determination coefficient checked the quality of the calibration model (R^2), root mean square error of cross-validation (RMSECV), Residual prediction deviation (RPD) and a bias value.

The external validation was summarized as % accuracy and standard deviation (SD). The equations were calculated for the validation step (Junhom et al., 2017).

$$\% \text{ Accuracy} = 100 - \% \text{ Error}$$

$$\% \text{ Error} = \text{Relative error} \times 100$$

$$\textit{Relative error} = \left| \frac{\text{Xmean} - \text{Xa}}{\text{Xa}} \right|$$

When Xmean = FT-IR predicted value and Xa = actual TVC value.

CHAPTER IV

RESULTS

4.1 Total viable counts (TVC) of bacterial spoilage in milk during incubation

Total viable counts for *B. cereus* and *P. aeruginosa* in monoculture and co-culture in milk after they had incubated at 37 °C were expressed as mean log of colony forming units per ml (cfu/ml). The results were shown in Table 4.1, Figure 4.2 and Figure 4.3. The co-culture grown on TSA medium provided different types of colonies regarding morphology, size, shape, colour, and texture (Nicolaou et al., 2011). Colonies of *B. cereus* grown on TSA were irregular, approximately 3-7 mm in diameter, opaque in colour and smooth. Colonies of *P. aeruginosa* were irregular, approximately 2 mm in diameter, transparent to light yellow, shiny in colour and smooth as depicted in Figure 4.1. The results obtained from conventional total viable count methods exhibited that the initial inocula of microbes were relatively low in milk samples. The initial inoculation numbers of all experiments were similar to mean log TVC of 3.74 and 3.39 for a pure culture of *B. cereus* and *P. aeruginosa*, respectively. Likewise, co-culture also showed the average log TVC of 3.60 and 3.00 for *B. cereus* and *P. aeruginosa*, respectively.

In the lag phase of *B. cereus* grown in both experiments during 0 to 60 min, the mean log TVC were 3.74-4.13 and 3.60-3.96 for mono and co-culture, respectively. The exponential growth phase increased significantly at 120 min and terminated at 720 min with mean log TVC of 5.07-7.97 (mono-culture) and 4.90-8.17 (co-culture). The stationary phases ended the final mean log TVC were 7.65 and 7.99 after 1,440 min or 24 h of growth for mono and co-culture, respectively.

In the lag phase of *P. aeruginosa* also showed in both experiments from 0 to 60 min, with mean log TVC of 3.39-3.59 and 3.00-3.35 for mono and co-culture, respectively. The exponential growth phase started obviously at 120 min and finished at 720 min with mean log TVC of 3.80-8.89 (mono-culture) and 4.00-7.65 (co-culture). The stationary phases reached the final mean log TVC were 9.02 and 8.19 after 1,440 min or 24 h of growth for mono and co-culture, respectively. These findings indicated that the presence of *B. cereus* in the co-culture interfered the growth of *P. aeruginosa* resulting in a decrease of a lower final mean log TVC of 8.19 at 24 h when compared to 9.02 in mono-culture.

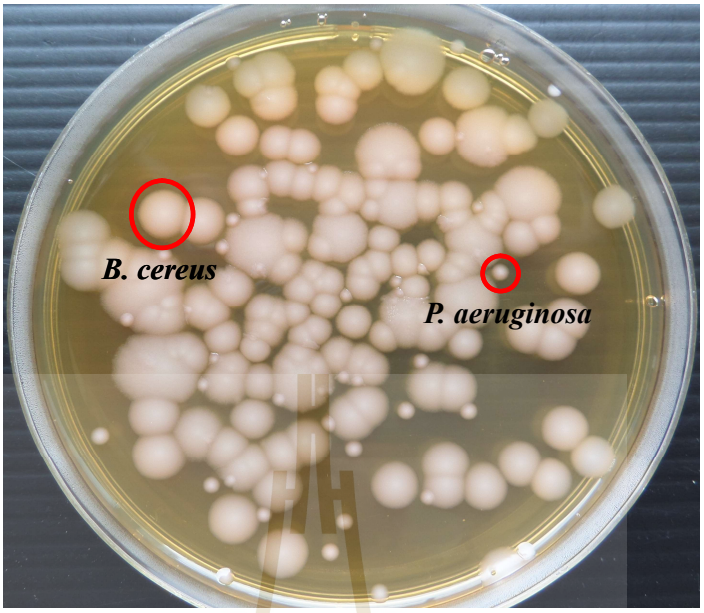

Figure 4.1 *B. cereus* and *P. aeruginosa* showed the difference in morphology of colonies from co-culture on tryptic soy agar at 37 °C after 20 h incubation.

Table 4.1 Mean log TVC for *B. cereus* growth at 37 °C in UHT milk, both in mono-culture, and co-culture with *P. aeruginosa*.

Storage Time (minutes)	Microbial analysis (log cfu/ml)			
	Pure culture		Mixed culture	
	B. cereus	***P. aeruginosa***	***B. cereus***	***P. aeruginosa***
0	3.74 ± 0.06^{a}	3.39 ± 0.12^{a}	3.60 ± 0.00^{a}	3.00 ± 0.00^{a}
60	4.13 ± 0.07^{b}	3.59 ± 0.16^{b}	3.96 ± 0.26^{a}	3.35 ± 0.49^{a}
120	5.07 ± 0.04^{c}	3.80 ± 0.45^{c}	4.90 ± 0.01^{a}	4.00 ± 0.00^{b}
150	5.00 ± 0.06^{c}	4.06 ± 0.03^{d}	6.02 ± 0.03^{b}	3.95 ± 0.36^{b}
180	6.14 ± 0.20^{d}	4.15 ± 0.21^{e}	6.27 ± 0.02^{b}	4.15 ± 0.21^{b}
210	6.36 ± 0.08^{d}	4.35 ± 0.49^{f}	7.20 ± 0.00^{b}	5.00 ± 0.00^{c}
240	6.19 ± 0.02^{d}	4.65 ± 0.07^{f}	7.24 ± 0.09^{c}	5.15 ± 0.21^{c}
300	6.45 ± 0.21^{d}	5.65 ± 0.07^{f}	7.30 ± 0.06^{d}	5.45 ± 0.21^{c}
360	7.45 ± 0.02^{e}	6.47 ± 0.26^{g}	7.52 ± 0.06^{e}	5.74 ± 0.06^{d}
420	7.79 ± 0.05^{f}	7.40 ± 0.00^{h}	7.78 ± 0.03^{f}	6.69 ± 0.30^{e}
480	7.85 ± 0.02^{f}	8.06 ± 0.01^{h}	7.97 ± 0.04^{g}	6.85 ± 0.21^{e}
720	7.97 ± 0.01^{f}	8.89 ± 0.14^{h}	8.17 ± 0.12^{h}	7.65 ± 0.05^{f}
960	7.90 ± 0.08^{f}	9.01 ± 0.09^{h}	8.02 ± 0.09^{h}	8.00 ± 0.06^{f}
1440	7.65 ± 0.07^{f}	9.02 ± 0.03^{h}	7.99 ± 0.12^{h}	8.19 ± 0.34^{g}

Mean values ± standard error.

A significant difference between each group was compared using ANOVA and Fisher' s least significant difference (LSD) *post hoc* test at a $p<0.05$.

abcdefgh, Mean log TVC within a column with the same superscripts are not statistically significant difference ($p<0.05$).

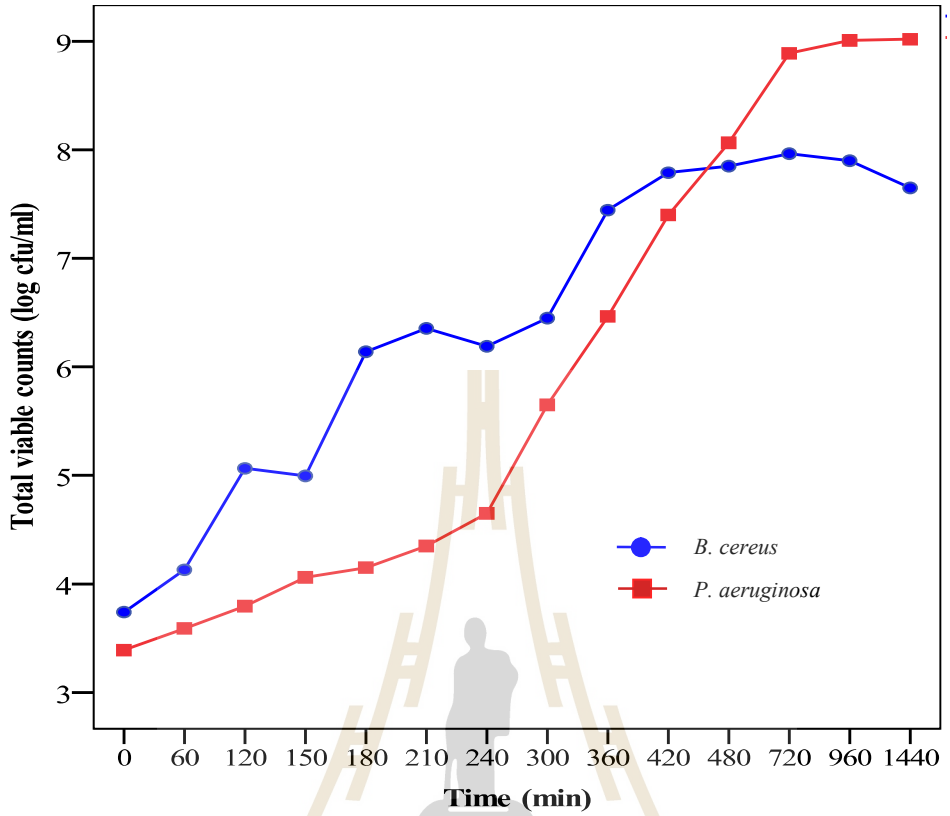

Figure 4.2 Mean logTVC of *B. cereus* and *P. aeruginosa* in UHT milk samples when they were inoculated as mono-culture at different times throughout 24 h. The average values were shown for each time-point.

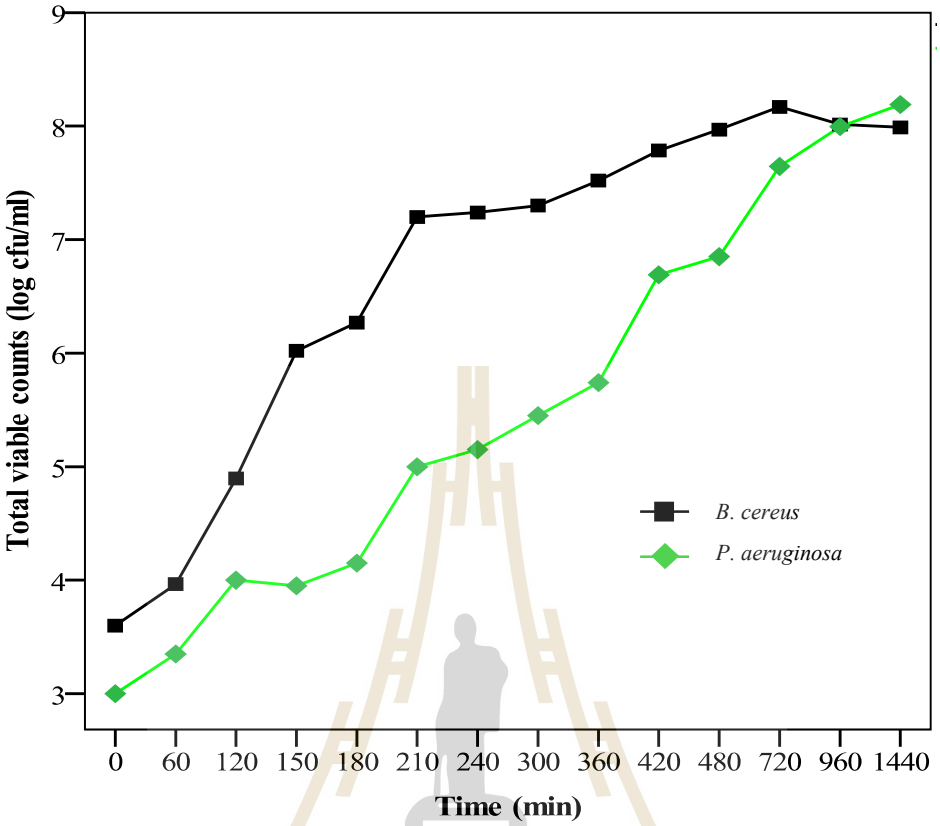

Figure 4.3 Mean logTVC of *B. cereus* and *P. aeruginosa* in UHT milk samples when they were inoculated as co-culture at different times throughout 24 h. The average values were shown for each time-point.

4.2 FT-IR microspectroscopic analysis for spoilage milk

4.2.1 FT-IR reflectance spectra

Vibrational micro-spectroscopic techniques are rapidly emerging as effective tools and have become more popular in studying the biological samples because these techniques investigate the vibrational modes of the molecules and provide spectra that are unique to the molecular properties and chemical nature of the species (Singh et al., 2012). The functional group of the biochemical composition of

the milk sample (Figure 4.4) showed representative spectra from FT-IR microspectroscopy at 480 min (8h). The FT-IR spectra of the two distinct bacterial species in mono-culture and co-culture, only some differences between spectra were seen. The representative FT-IR spectra revealed similar characteristics and located in three regions: lipids (3000-2800 cm^{-1}), proteins (1800-1500 cm^{-1}) and carbohydrates (1200-900 cm^{-1}) in all milk samples. Accordingly, for the FT-IR spectra measurement, the weak absorbance < 0.35 units or high absorbance > 1.2 units were ignored for spectral pre-processing.

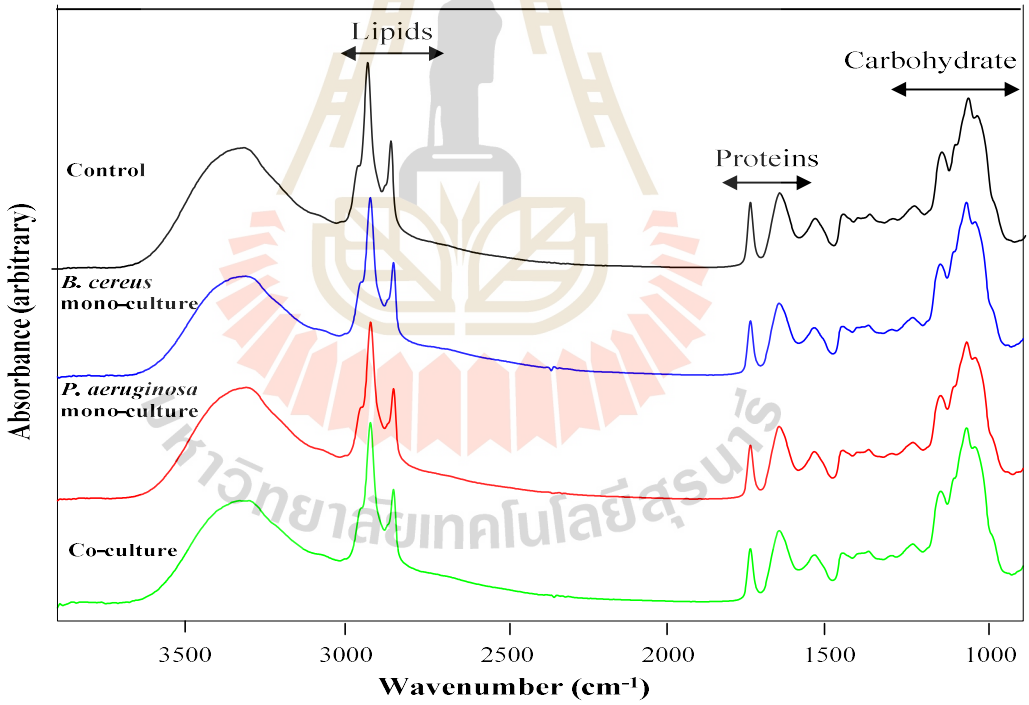

Figure 4.4 FT-IR spectra of control and milk sample after inoculation with *B. cereus* and *P. aeruginosa* in mono-culture and *B. cereus* and *P. aeruginosa* in co-culture at 480 min (8h). Spectra features could be observed.

Figure 4.5 showed the representative FT-IR reflectance spectra (4000 to 900 cm^{-1}) of UHT milk (control) and UHT milk sample after inoculation. *B. cereus* in mono-culture (Figure 4.5A), *P. aeruginosa* in mono-culture (Figure 4.5B) and co-culture (Figure 4.5C) at 37 °C for control (0 h), 480 min (8 h) and 960 min (16 h), respectively. The qualitative similarity both of *B. cereus*, *P. aeruginosa* in mono-culture and co-culture, the spectra corresponding to UHT milk at 480 min (8 h) and 960 min (16 h) showed higher signal in the region between at 3000-2800 cm^{-1} and 1800-1500 cm^{-1}, compared with control UHT milk. The absorbance at 1200-900 cm^{-1} of UHT milk at 480 min (8 h) were higher changed in vibration when compared with the spectrum between control UHT milk, but the absorbance at 1200-900 cm^{-1} of UHT milk at 960 min (16 h) showed slightly lower signal than UHT milk at 480 min (8 h). The difference of metabolic fingerprints of UHT milk with bacterial spoilage had increased at the signal of lipids, proteins and had decreased at the signal of carbohydrate utilization by the fermentative metabolism resulting in a compositional change of milk.

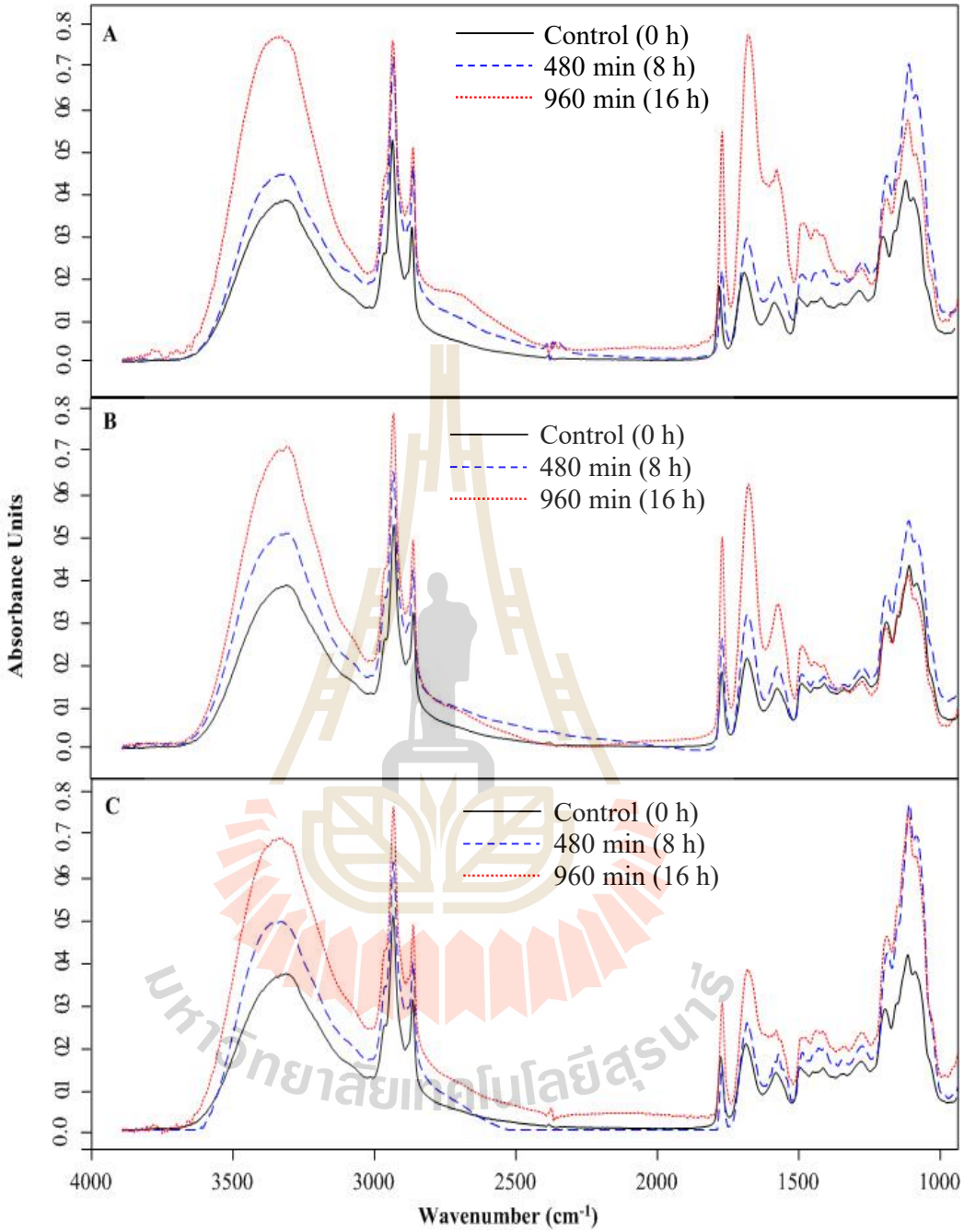

Figure 4.5 Representative FT-IR reflectance spectra (4000 to 900 cm^{-1}) of milk samples after inoculation with *B. cereus* in mono-culture (A), *P. aeruginosa* in mono-culture (B) and co-culture (C) at 37 °C for control (0 h), 480 min (8 h) and 960 min (16 h).

4.2.2 Cluster analysis

B. cereus was grown in milk at 200 rpm, 37 °C for 24 h. FT-IR microspectroscopy was investigated for its ability to generate a significant change in metabolic fingerprints of the milk during the growth of *B. cereus* (gram-positive bacteria). Graphic inspection of the spectra obtained from PCA analysis of *B. cereus* in mono-culture revealed any differences between the FT-IR spectra cluster analysis and the PCA results for the relationship between the FT-IR and the total viable counts in milk as shown in Figure 4.6a. From the PCA results plot, it could be seen that the spectra for the first control group in the region on the between positive and negative right of Figure 4.6a. The spectra of samples incubated at the 360 min time point were distinctly different from the control group, 480 min group tended to spread towards the negative left-hand side, and then 960 min group were upward rising. The total viable counts had the mean log TVC equal to 7.45 (360 min), 7.79 (480 min) and 7.90 (960 min), respectively.

PCA analysis was constructed by using the second derivative, normalising spectra with EMSC, over the spectral regions between 3000-2800 cm^{-1} and 1800-900 cm^{-1}. The PCA separated from each group corresponding to the results of loading (Figure 4.6b). The typical second loading plot separated the clusters along PC1 which can be explained by distinct PC1 loadings in the region at 3000-2800 cm^{-1}. These regions were assigned to the stretching mode of CH_2 and CH_3 (2957, 2923, 2852 cm^{-1}) of fatty acids. These absorptions were also explained dominant peak at the fatty acid region of 360, 480 and 960 min group showed lipid increase when compared with control, respectively (Figure 4.6c).

The representative second loading showed the ester bond of fatty acid at 1745 cm^{-1}, amide I band at 1652 cm^{-1} assigned to the α-helix structure of the protein. 1172, 1118 and 1074 cm^{-1} assigned to a polysaccharide (stretching mode of C-O-C) as shown in Figure 4.6d.

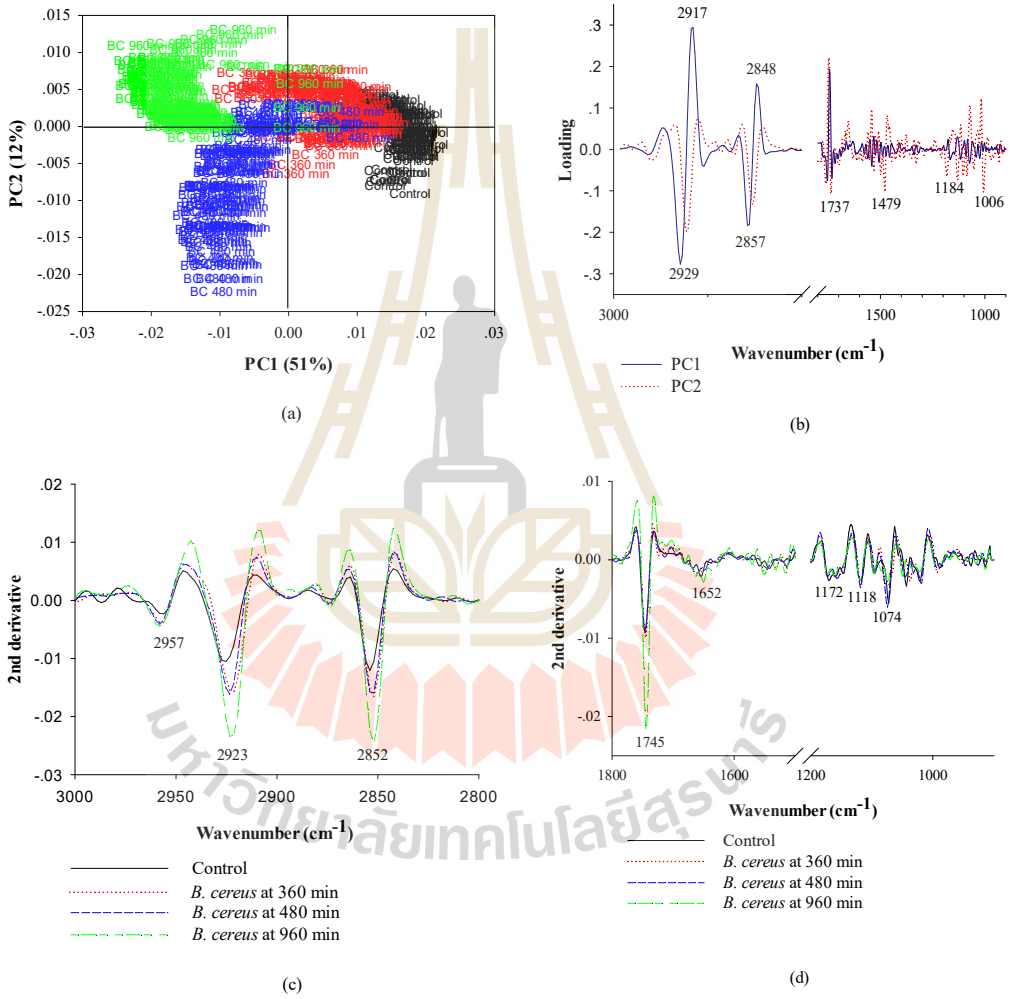

Figure 4.6 PCA results of control and *B. cereus* in mono-culture at 360, 480 and 960 min of the 1st (PC1) and 2nd (PC2). (a) score scatter plot, (b) the loading plot, (c) the representative 2nd derivative transformation spectra (3000-2800 cm^{-1}), (d) the representative 2nd derivative transformation spectra (1800-900 cm^{-1}).

The different trend was also seen in the PCA results from the collected from the different bacteria (gram-negative bacteria) with the excellent separation between time-points at control as well as 360, 420 and 720 min group of *P. aeruginosa* in mono-culture (Figure 4.7a). The Figure 4.7b showed loading plot to classify a group of treatment corresponded to PCA. The typical second loading plot separated of clusters along PC1 which can be explained by distinct PC1 loadings in the region at 3000-2800 cm^{-1} and 1745 cm^{-1}. These regions represented by the stretching mode of CH_2 and CH_3 (2960, 2923, 2852 cm^{-1}) of fatty acids (Figure 4.7c) and an ester bond of fatty acid together (Figure 4.7d).

The representative second loading of spectra was shown in Figure 4.7d the amide I band at 1652 cm^{-1} that were assigned to the α-helix structure of the protein. 1118, 1077 and 1022 cm^{-1} assigned to a polysaccharide (stretching mode of C-O-C). The metabolic fingerprints of *P. aeruginosa* in mono-culture illustrated among dominant peak at the fatty acid region of 360, 420 and 720 min group showed lipid increase when compared with control, respectively and same to *B. cereus* in mono-culture.

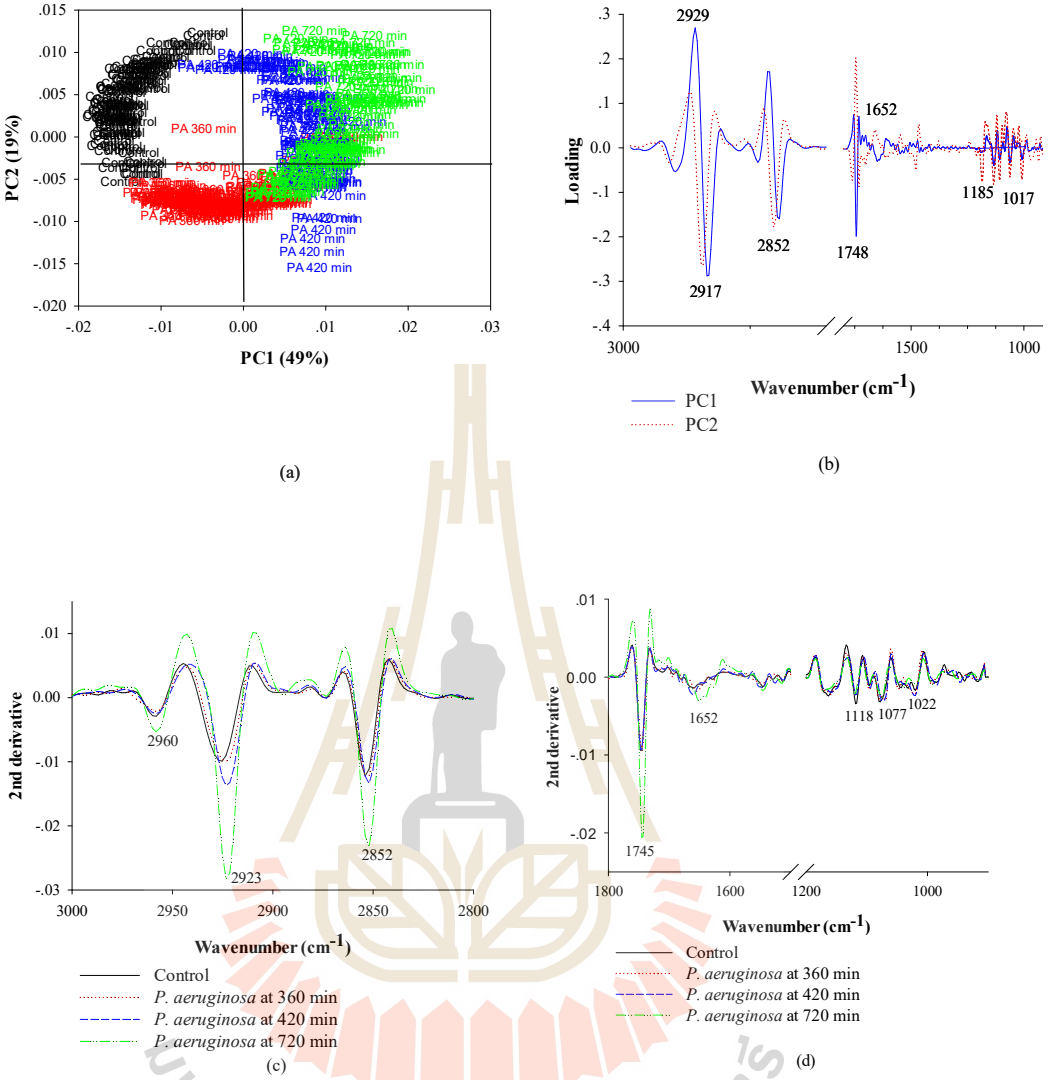

Figure 4.7 PCA results of control and *P. aeruginosa* in mono-culture at 360, 420 and 720 min of the 1st (PC1) and 2nd (PC2). (a) score scatter plot, (b) the loading plot, (c) the representative 2nd derivative transformation spectra (3000-2800 cm^{-1}), (d) the representative 2nd derivative transformation spectra (1800-900 cm^{-1})

The co-culture at 200 rpm, 37 °C and 24 h. The PCA showed separated with each group (Figure 4.8a) corresponding to the results of loading (Figure 4.8b). The second loading plot separated of clusters along PC1 which could be explained by distinct PC1 loadings in the regions at 3000-2800 cm^{-1} and 1752 cm^{-1}. These regions were stretching mode of CH_2 and CH_3 (2959, 2922, 2852 cm^{-1}) of fatty acids and an ester bond. (Figure 4.8c and 4.8d). These absorptions were also explained dominant peak at the fatty acid region of 150 and 360 min group showed lipid increase when compared with control, respectively. Lipid of 1,440 min group would be decreased lower than control (Figure 4.8c).

The second loading showed the amide I band at 1659 cm^{-1} that were assigned to the α-helix structure of the protein. The 1162, 1116 and 1074 cm^{-1} represented polysaccharide (Figure 4.8d). The metabolic fingerprints of co-culture illustrated the different trend which was also seen in PCA from mono-culture from FT-IR data collected from any bacteria.

Overall these PCA results showed that the sample variation was distinct at the early initiation of the bacterial growth for mono and co-culture. However, the estimation of bacterial numbers would rather accurate than the growth time, these results demonstrate that PLS should be determined accurately for bacterial quantification from these FT-IR data.

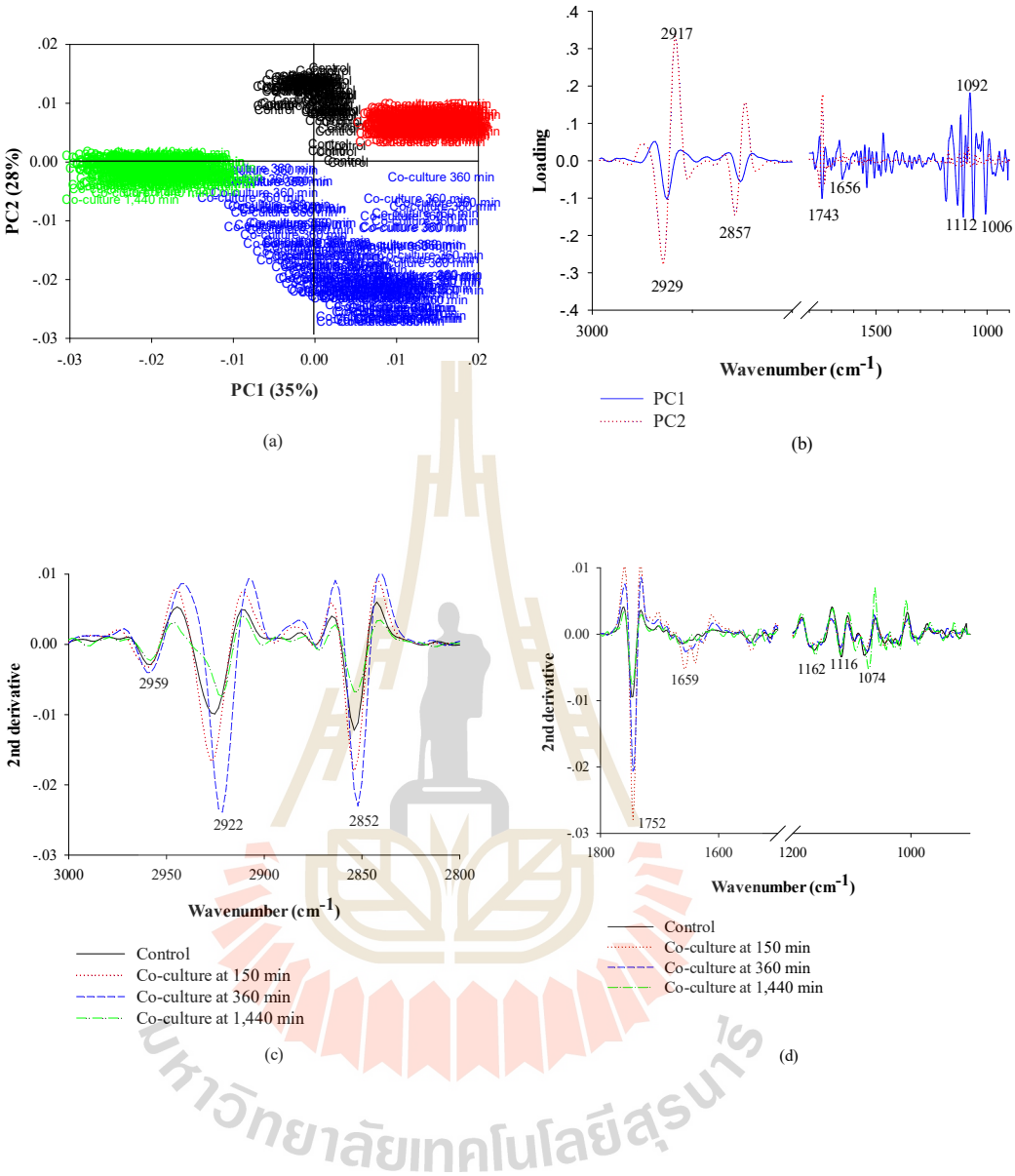

Figure 4.8 PCA results of control, *B. cereus* and *P. aeruginosa* in co-culture at 150, 360 and 1,440 min of the 1st (PC1) and 2nd (PC2). (a) score scatter plot, (b) the loading plot, (c) the representative 2nd derivative transformation spectra (3000-2800 cm^{-1}), (d) the representative 2nd derivative transformation spectra (1800-900 cm^{-1}).

4.3 Quantification model of bacteria in spoilage milk using the PLS analysis

The results of cross-validation PLS models that were performed using a PLS model from the FT-IR spectra of UHT milk inoculated with *B. cereus* and *P. aeruginosa* for quantification of bacterial spoilage in milk were summarized in Table 4.2. The regression coefficient (R^2) gave the accuracy of the model's root mean squares error for the cross-validation (RMSECV) and residual prediction deviation (RPD). The good PLS quantification model for total viable counts prediction was high values which R^2 was ≥0.95, and RMSECV was ≤1. The closer R^2 to 1 would explain the high correlation between the actual total viable counts (reference method value) and the predicted total viable counts value (FT-IR predicted value). Residual prediction deviation (RPD) was the ratio of the standard deviation (SD) of the reference values and the standard error of prediction (SEP). The RPD value characterised the performance of the quantification model. It was acceptable, good and excellent when the RPD were > 3, > 5 and > 10, respectively. The systematic errors of the model revealed by the bias value refer to the difference between the average and reference values. The active model should have a low bias (Junhom et al., 2017)). The total of 15,250 spectra was used for model calibration. Graphs were used to show the comparison between the actual total viable counts and the predicted total viable counts value of *B. cereus* and *P. aeruginosa* that inoculated into the UHT whole milk with mono and co-culture in Figure 4.9 and 4.10. Table 4.3 and 4.4 showed very good prediction with high accuracy in models, according to % accuracy

(94-99%) in mono-culture and (95-99%) in co-culture. These results showed that the PLS model from FT-IR microspectroscopy combined with PLS applied to the wavenumbers in the lipid (3000-2800 cm^{-1}), protein (1800-1500 cm^{-1}) and polysaccharide (1200-900 cm^{-1}) regions were reliable for the quantification of spoilage milk.

Table 4.2 Results of PLS carried out on FT-IR spectra (~3000-2800 cm^{-1} and 1800-900 cm^{-1}) and microbial data obtained by total viable counts.

Model	Calibration samples	RMSECV	R^2	RPD	Bias
B. cereus (mono-culture)	78	0.24	0.972	5.98	-0.00457
P. aeruginosa (mono-culture)	90	0.35	0.971	5.84	-0.0137
B. cereus (co-culture)	113	0.16	0.982	7.35	-0.000646
P. aeruginosa (co-culture)	97	0.22	0.981	7.2	0.000309

R^2 = Coefficient of determination.

RPD = Residual prediction deviation.

RMSECV = Root mean square error of cross-validation.

Bias = mean value of deviation or systematic error.

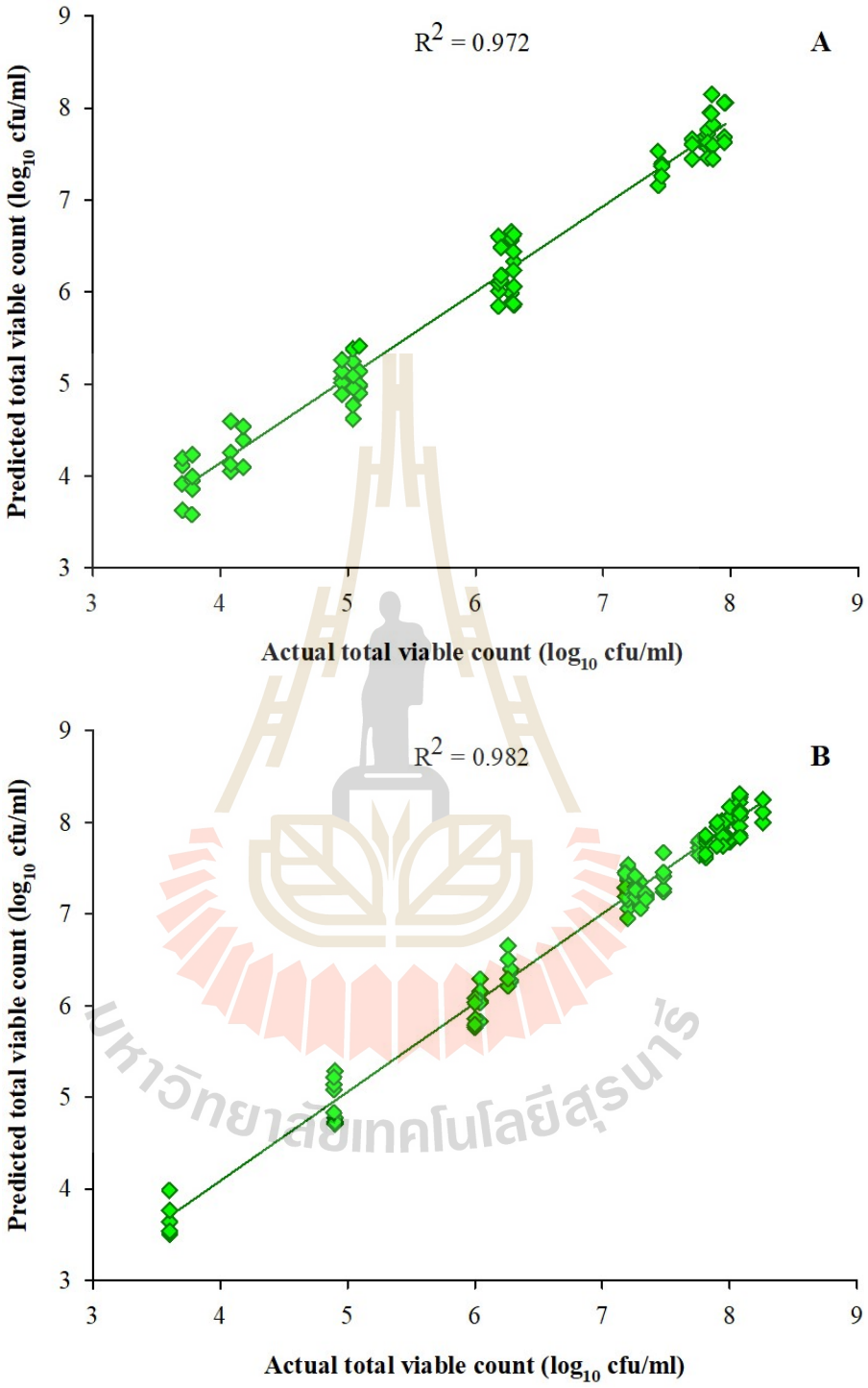

Figure 4.9 PLS calibration models for the relationship between actual and predicted total viable count for *B. cereus,* (A) mono-culture and (B) co-culture. FT-IR predicted using the second derivative.

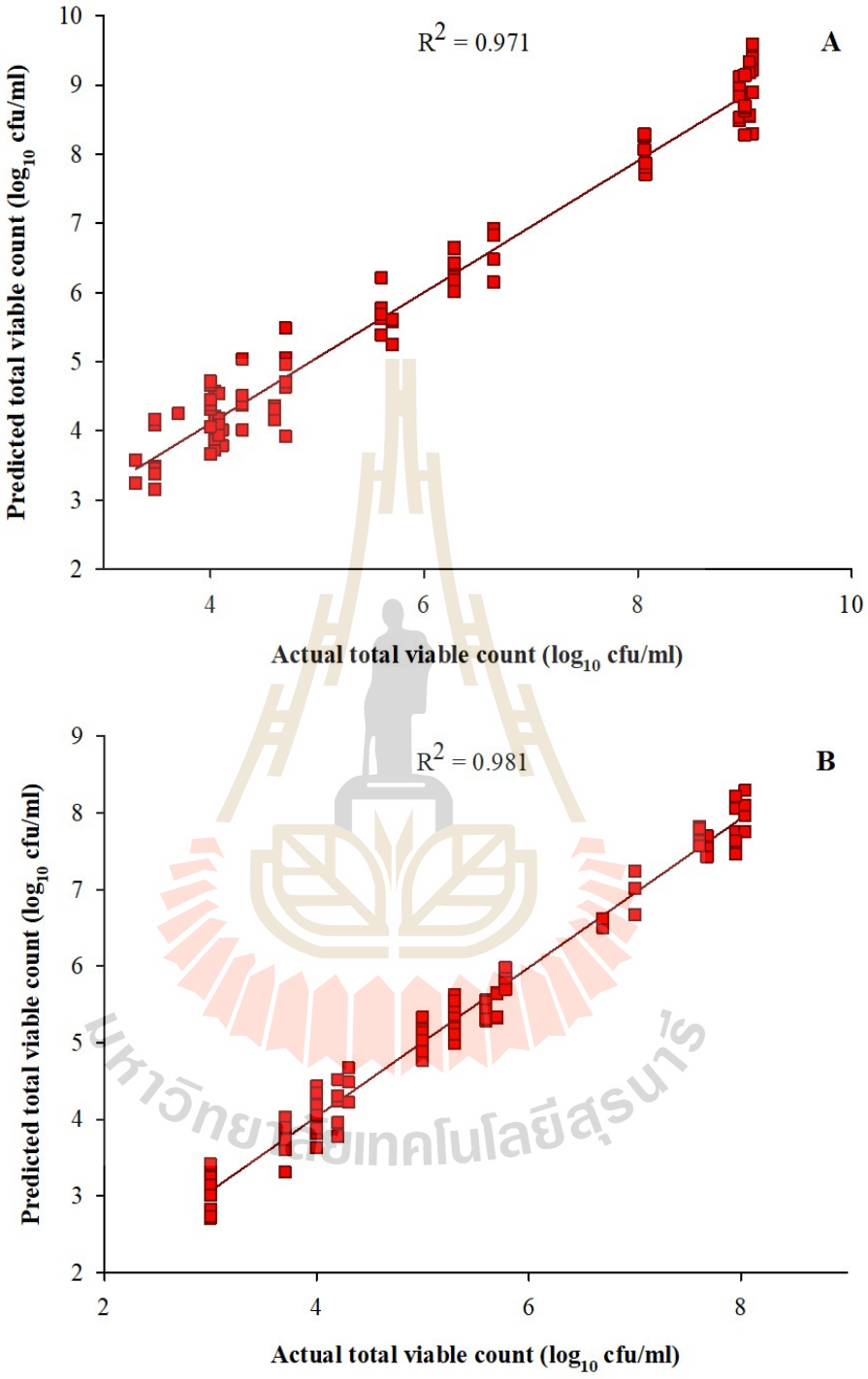

Figure 4.10 PLS calibration models for the relationship between actual and predicted total viable count for *P. aeruginosa,* (A) mono-culture and (B) co-culture. FT-IR predicted using the second derivative.

Table 4.3 The PLS model obtained from 2 data pre-processes were used to compare the actual TVC and the predicted (FT-IR predicted value) for *B. cereus* concentration in mono and co-culture.

Model	Mean actual Total viable counts	Mean of predicted Total viable counts ± SD	% Accuracy
B. cereus (mono-culture)	3.74	3.939 ± 0.228	94.68
	4.13	4.290 ± 0.218	96.12
	5.0	5.021 ± 0.152	99.58
	6.19	6.190 ± 0.231	99.99
	7.79	7.630 ± 0.109	97.95
B. cereus (co-culture)	3.60	3.687 ± 0.194	97.59
	4.90	4.970 ± 0.236	98.57
	6.02	5.989 ± 0.173	99.49
	6.27	6.352 ± 0.156	98.69
	7.20	7.253 ± 0.167	99.26

Table 4.4 The PLS model obtained from 2 data pre-processes were used to compare between the actual TVC and the predicted (FT-IR predicted value) for *P. aeruginosa* concentration in mono and co-culture.

Model	**Mean actual Total viable counts**	**Mean of predicted Total viable counts ± SD**	**% Accuracy**
P. aeruginosa	3.39	3.506 ± 0.326	96.59
(mono-culture)	4.06	4.121 ± 0.289	98.49
	5.65	5.643 ± 0.286	99.88
	6.47	6.480 ± 0.337	99.83
	8.06	7.970 ± 0.237	98.89
P. aeruginosa	3.35	3.193 ± 0.304	95.32
(co-culture)	4.00	4.014 ± 0.224	99.65
	5.45	5.446 ± 0.162	99.92
	6.85	6.780 ± 0.288	98.98
	7.65	7.627 ± 0.133	99.70

CHAPTER V

DISCUSSION AND CONCLUSION

Fourier transform infrared spectroscopy (FT-IR spectroscopy) or the mid-IR region includes the wavelength range of 4000 to 400 cm^{-1}. The fundamental principle of FT-IR technique is especially various organic functional groups absorb infrared light at specific wavelengths, such as proteins, carbohydrates, lipids and nucleic acids. The evaluation of the data involves multivariate statistical analysis. So, rapid spectroscopic screening of milk for the enumeration of pathogenic microorganisms has reformed a powerful tool for determining microbial food safety and public health security. FT-IR microspectroscopy is now possible to analyse a wide range of metabolites present in food and produce by microorganisms. It can detect a significant amount of the biochemical fingerprint data quickly with rather a low cost. Achieving quantitative estimations of many biochemical components are possible. FT-IR microspectroscopy has been already applied to various food pathogens and spoilage microorganisms because it is rapid, sensitive and comparatively inexpensive (Lin et al., 2005; Nicolaou and Goodacre, 2008; Schmidt et al., 2012; Wang et al., 2011). It has also been used to rapidly monitor meat spoilage (Ellis et al., 2002; Panagou et al., 2011; Papadopoulou et al., 2011). Recently, this technique showed detection of heat-induced conformational rearrangements of proteins, protein-protein

and protein-lipid interactions in UHT milk which was studied with accelerated shelf-life protocols (Grewal et al., 2017).

The study revealed that FT-IR microspectroscopy could separate bacterial spoilage in milk. It could be applied to UHT milk for the rapid discrimination and quantification of mono-culture of bacteria. Beside, *B. cereus* and *P. aeruginosa* were evaluated by FT-IR microspectroscopy as a method for the quantification of bacterial populations in mono-culture and co-culture based on compositional changes in milk derived from their metabolic activities. Three inoculation experiments were examined; firstly with *B. cereus* ATCC 11778 the secondly with *P. aeruginosa* ATCC 15442 and thirdly with co-cultures of both *B. cereus* ATCC 11778 and *P. aeruginosa* ATCC 15442. Inoculated milk samples were incubated at 200 rpm shaker at 37 °C for 24 h. The samples were taken at 0, 60, 120, 150, 180, 210, 240, 300, 360, 420, 480, 720, 960 and 1,440 min from all experiments. The samples were then determined the total viable counts by using the spread plate count technique. The same samples were also analyzed for their infrared spectrums using the aliphatic C-H stretching region of fatty acid (3000-2800 cm^{-1}), the amide I and II bands of proteins (1800-1500 cm^{-1}) and lactose C-C, CO of polysaccharides (1200-900 cm^{-1}). The absorbance spectra with mathematic pretreatment were used to correlate variable by principal component analysis (PCA). PCA inspected the spectra, checked for outliers. Moreover, FT-IR measurements were performed with microorganisms sampling at different growth phases. Measurements and data analysis were also applied to the growth of bacteria in co-culture. The results revealed that the growth of *B. cereus* was almost entirely

different in mono-culture and co-culture at different growth phases compared to *P. aeruginosa*. Partial least square (PLS) was conducted for the prediction model to enumerate the total viable counts in milk. It was found that the model developed by mono-culture and co-culture gave a good prediction performance. The best model was developed after spectra pretreatment of the second derivative, and they provided a coefficient of determination (R^2) and root mean squares error for the cross-validation (RMSECV), respectively. Firstly, the model of *B. cereus* in mono-culture gave R^2 of 0.972 and RMSECV of 0.24. Secondly, the model of *P. aeruginosa* in mono-culture gave R^2 of 0.971 and RMSECV of 0.35. Thirdly, the model of *B. cereus* in co-culture gave R^2 of 0.982 and RMSECV of 0.16. Finally, the model of *P. aeruginosa* in co-culture gave R^2 of 0.981 and RMSECV of 0.22. Most actual total viable counts were predicted with high accuracy (94-99%) such that the linear correlation between the actual and predicted FT-IR values was nearly perfect (slope~1). A PLS model was performed, the results showed an excellent linear relationship between FT-IR predicted value and actual TVC value and extensively covered the TVC values. It could also be used efficiently for the detection of the bacterial load in milk and quantification of the specific spoilage bacteria in milk. The accuracy of the total viable count measurements on the basis of models for calibration of samples in terms of bias. The standard error of prediction (SEP) is of the same order of magnitude as reported in the pioneering FT-IR work of Nicolaou and Goodacre (2008), they used FT-IR techniques and chemometrics in pasteurized milk authorized to perish naturally during storage at 15 °C, described that metabolic fingerprinting using FT-IR

spectroscopy had a very good application potential in the dairy production as a rapid and non-invasive technique of bacterial spoilage detection. The assessment of the growth interactions between *Staphylococcus aureus* and *Lactococcus lactis* subsp. *cremoris* in milk had been also used successfully in the analysis of the rapid discrimination and enumeration (Nicolaou et al., 2011). Similar were the conclusions reached by Lianou et al. (2017) in a study assessing the performance of FT-IR spectroscopy as a rapid and non-invasive technique for the automated microbiological quality evaluation of pasteurised vanilla cream. In addition, PLS was used for prediction of fat, crude protein, lactose and urea after preprocessing IR data. Excellent results were obtained for prediction of crude protein, lactose and urea content (R^2 > 0.99, 0.98 and 0.86 respectively) in raw and homogenized milk using micro-attenuated total reflection IR spectroscopy (Aernouts et al., 2011).

The performance of the calibration approach presented here has not yet been investigated in the practice of a dairy or animal recording laboratory so that FT-IR microspectroscopy constitutes a promising method for the real-time assessment of the microbial quality of pasteurised milk. This work is just a preliminary study since. *B. cereus* was selected as the representative of the gram-positive bacterium and *P. aeruginosa* be the representative of gram-negative one. Since these two bacteria are psychrotrophic bacteria, thus they are commonly found to contaminate in milk. However, there are other bacteria by Notification of the Ministry of Public Health (No.364) B.E.2556 (2013): standards for pathogenic microorganisms in food such as *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* which can also be found in contaminated milk. Therefore, for the further study, the calibration spectra models of other bacteria that can be found in contaminated milk both mono-culture

and co-culture should be developed to cover more relative bacteria in milk and utilize to determine bacteria that associated in milk comprehensively.

The results discussed above will support the possible implementation of FT-IR microspectroscopy to identify and quality milk spoilage. In investigation together with total viable counts of *B. cereus* and *P. aeruginosa* can establish the calibration model for the infrared determination of milk samples. More information on other bacterial calibration spectra models is needed to provide accurate and rapid result for predicting microbial loads in pasteurised milk.

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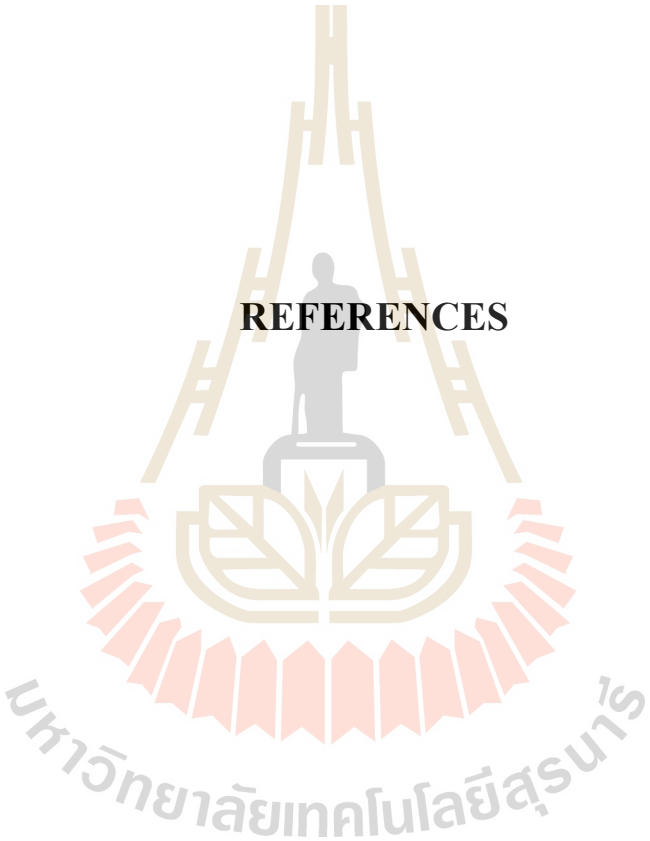

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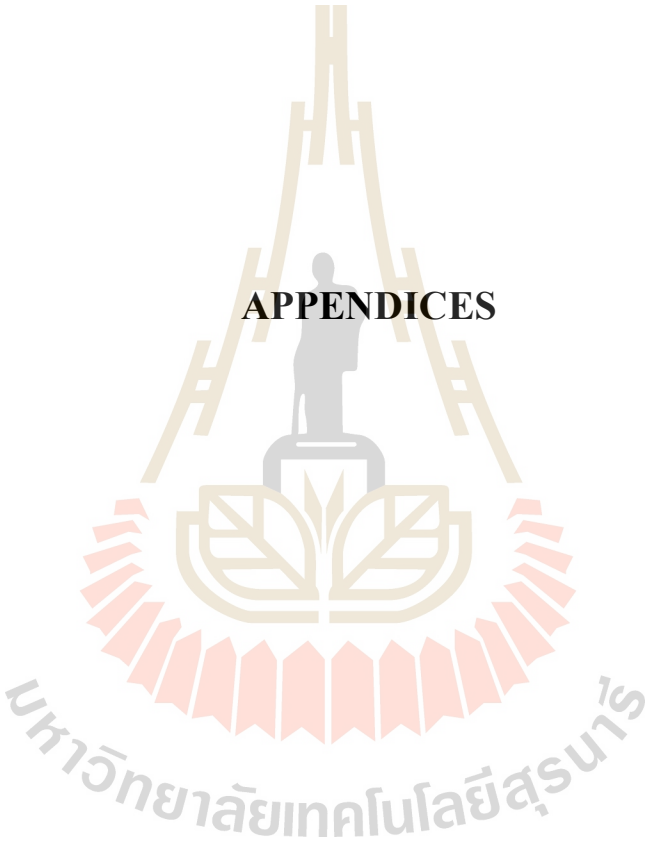
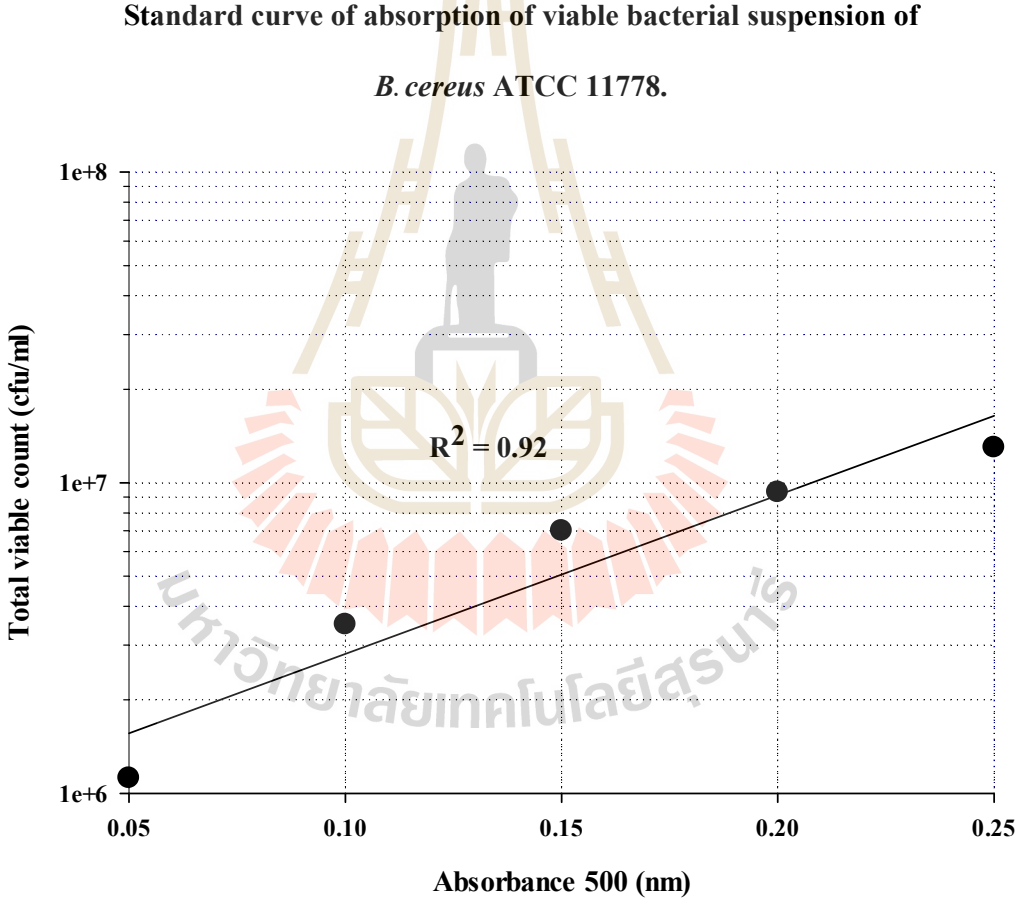
APPENDICES



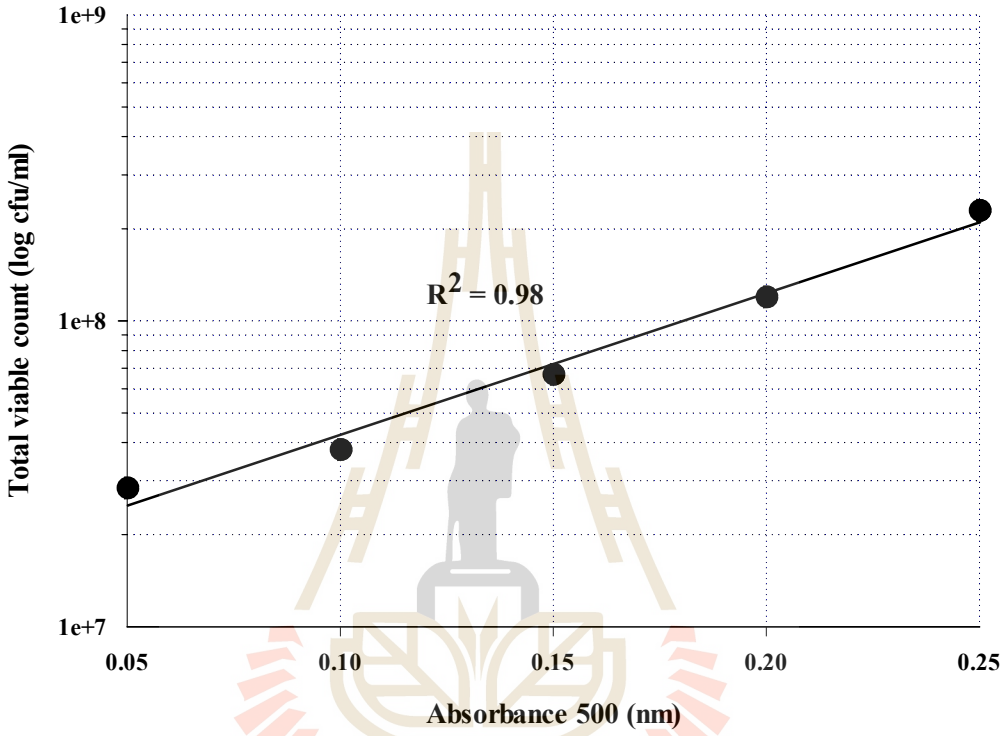
APPENDIX A

BACTERIAL SUSPENSION STANDARD CURVE

A1. Standard curve of absorption of viable bacterial suspensions



Standard curve of absorption of viable bacterial suspension of *P. aeruginosa* ATCC 15442.



APPENDIX B

PREPARATION OF REAGENTS

B1. Nutrient agar

The Merck nutrient agar was used for cultivation of a stock culture on agar slopes.

The formula was:	Grams/Litre
Peptone from meat	5.0
Meat extract	3.0
Agar	12.0

B2. Nutrient broth

The Merck nutrient broth

The formula was:	Grams/Litre
Peptone from meat	5.0
Meat extract	3.0

B3. Tryptic soy agar

The Merck tryptic soy agar was used for enumeration of *B. cereus* and *P. aeruginosa*

The formula was:	Grams/Litre
Peptone from casein	15.0
Peptone from soymeal	5.0
Sodium chloride	5.0
Agar	15.0

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