Faculty of Resource Science and Technology

Detection and Molecular Characterization of *Bacillus cereus* Isolated from Sago Processing Plants in Sarawak

Jasmin binti Jaraee

Master of Science
2018

UNIVERSITI MALAYSIA SARAWAK

Grade: _____

Please tick (√)

Final Year Project Report	☐
Masters	☑
PhD	☐

DECLARATION OF ORIGINAL WORK

This declaration is made on the 15 day of ~~August~~ SEPTEMBER 2018.

Student's Declaration:

I JASMIN BINTI JARA'EE, 1502198, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY

(PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, DETECTION AND MOLECURAR CHARACTERISATION OF BACILLUS CEREUS IN SAGO PROCESSING PLANTS IN SARAWAK is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

28/9/2018
Date submitted

JASMIN BINTI JARA'EE (1502198)
Name of the student (Matric No.)

Supervisor's Declaration:

I LESLEY MAURICE BILUNG (SUPERVISOR'S NAME) hereby certifies that the work entitled, DETECTION AND MOLECULAR CHARACTERISATION OF BACILLUS CEREUS IN SAGO PROCESSING PLANTS IN SARAWAK (TITLE) was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of MASTER OF SCIENCE (MICROBIOLOGY) (PLEASE INDICATE THE DEGREE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: LESLEY MAURICE BILUNG Date: 26/9/18
(Name of the supervisor)

I declare this Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

Validation of Project/Thesis

I therefore duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitise the content to for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student itself neither third party on this Project/Thesis once it becomes sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student's signature _____ 27.9.2018
(Date)

Supervisor's signature: 26.9.2018
(Date)

Dr Maurice Bilung
Pensyarah Kanan Industri
Program Biosinologi Sungai
Fakulti Sains dan Teknologi Sumber
Universiti Malaysia Sarawak

Current Address:
132 A, KAMPUNG PANGLIMA SEMAN ULU, 93050, KUCHING SARAWAK.

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument was duly prepared by The Centre for Academic Information Services]

Detection and Molecular Characterization of *Bacillus cereus* Isolated from Sago Processing Plants in Sarawak

Jasmin binti Jaraee

A thesis submitted

In fulfilment of the requirements for the degree of Master of Science (Microbiology)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2018

DECLARATION

The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

_____

Name: Jasmin binti Jaraee

Matric number: 15020198

30 January 2018

ACKNOWLEDGEMENT

All praises to Allah the Almighty for the blessings, strength and love He has given to me and for granting me the strength, determination and patience to accomplish my master degree. A special thanks and sincere appreciation I dedicated to my respected supervisor, Dr. Lesley Maurice Bilung and my co-supervisors, Assoc. Prof. Dr. Cirilo Hipolito Nolasco and Dr. Micky Vincent for their advices, guidance, encouragement as well as skills and knowledge sharing throughout my study. Deepest gratitude and a big thank you to my family who had supported me morally as well as financially in time of difficulties. I am also deeply indebted to the members of Microbiology Laboratory especially Pui Chai Fung, Jennifer Jalan, Ahmad Syatir Mohd Tahar, Auns Mohamed and Grace Bebey for all their help, moral support and encouragement along my study journey. Not to forget, the lab assistants and UNIMAS driver for their help and service especially during sampling trips. Last but not least, thank you to everyone who helped and supported me directly and indirectly. Without your support, I would never be able to finish this project.

ABSTRACT

Sago processing industries are well-established and high potential industries in Sarawak. However, the contamination of bacteria might deteriorate its quality and become a concern to the public health. This study aimed to detect, quantify and characterize *Bacillus cereus* in sago processing in Sarawak, Malaysia. *B. cereus* was isolated from two selected sago processing mills in Sarawak. The prevalence and concentration of *B. cereus* in this study were determined firstly using selective agar and followed by using specific Polymerase Chain Reaction (PCR) by targeting specific virulence gene, haemolysin (*hly*) gene. A total of 120 samples consist of bark swab, sago pith, starch slurry, sago milk, sago flour and sago effluent were collected from each processing step in sago mills. It was revealed that *B. cereus* were present in 35% (42/120) of the samples. These isolates were subjected to molecular typing by using Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC PCR) and Pulsed Field Gel Electrophoresis (PFGE). Both molecular typing method showed heterogeneity of the *B. cereus* strain. Susceptibility of all isolates towards 14 antibiotics was assessed using disk diffusion assay. *B. cereus* isolates were uniformly resistant to penicillin and ampicillin whereas *B. cereus* isolates were uniformly susceptible to imipenem and norfloxacin. Multiple antibiotic resistance (MAR) index were calculated based on the antibiotic resistance results. The MAR index for all isolates were also varies, ranged from 0.083 to 0.750. This study is useful in developing appropriate intervention strategies and establishing food safety standards in sago processing in Sarawak thus contribute in lowering the disease burden and assist in providing safer food to the society.

Keywords: *B. cereus*, sago processing, detection, polymerase chain reaction, molecular characterization, antibiotic susceptibilities test

Pengesanan dan Pencirian Molekul Bacillus cereus yang Diasingkan dalam Kilang Pemprosesan Sagu di Sarawak

ABSTRAK

Industri pemprosesan sagu merupakan industri yang dikenali dan berpotensi tinggi di Sarawak. Walau bagaimanapun, pencemaran bakteria akan mengurangkan kualitinya dan menjadi isu kepada kesihatan awam. Kajian ini bertujuan untuk mengesan, mengira dan mencirikan B. cereus dalam pemprosesan sagu di Sarawak. B. cereus diasingkan daripada dua kilang pemprosesan sagu yang terpilih di Sarawak. Kelaziman dan penumpuan B. cereus dalam kajian ini dikaji dengan menggunakan agar selektif dan kemudian diuji dengan reaksi polymeras berantai spesifik untuk mengekspresikan gen sasaran iaitu gen hemolaisin. Sejumlah 120 sampel yang terdiri daripada olesan batang, empulur sagu, buburan kanji, susu sagu, tepung sagu dan efluen sagu diperolehi daripada setiap peringkat pemprosesan kedua-dua kilang. Ia menunjukkan bahawa B. cereus wujud di dalam 35% (42/120) sampel. Isolat ini tertakluk kepada pencirian molekul dengan menggunakan ERIC-PCR dan PFGE. Hasil daripada kedua-dua pencirian molekul ini menunjukkan kepelbagaian jenis B. cereus. Sensitiviti kesemua isolat terhadap 14 antibiotik ditaksir menggunakan B. cereus menunjukkan kerintangan kepada penisilin dan ampisilin serta sensitif kepada imipenum dan norfloxazin. Kajian ini berguna dalam membangunkan strategi untuk langkah pembaikan serta menghasilkan piawaian keselamatan makanan dalam pemprosesan sagu di Sarawak sekaligus menyumbang dalam mengurangkan beban penyakit dan membantu ke arah penyediaan makanan yang selamat kepada masyarakat.

Kata kunci: *B. cereus, pemprosesan sagu, pengesanan, tindak balas polimeras berantai, pencirian molekul, ujian sensitiviti antibiotik.*

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF ABREVIATIONS

µL	Microliter
µm	Micrometer
ºC	Degree Celsius
%	Percent
AFLP	Amplified fragment length polymorphism
AGE	Agarose gel electrophoresis
AOAC	Association of Official Analytical Chemists
AST	Antibiotic susceptibility test
CDC	Centre for Disease Control
CFU	Colony forming unit
ddH_2O	Double distilled water
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
ERIC	Enterobacteria repetitive intergenic consensus sequences
EtBr	Ethidium bromide
FDA	Food and Drug Association
FSIS	Food Safety and Inspection Service
ISO	International Organization of Standardization
HBL	Haemolysin BL
kb	Kilo base
kDa	Kilo Dalton

MAR	Multiple antibiotic resistant
$MgCl_2$	Magnesium Chloride
mL	Milliliter
MLST	Multilocus sequence typing
Mm	Milimeter
mM	Milimolar
MPN	Most probable number
MSRV	Modified Semisolid Rappaport-Vassiliades
MYP	Mannitol-Egg Yolk-Polymyxin
NCBI	National Center for Biotechnology Information
n.d	No date
NGFIS	Netherlands Government Food Inspection Services
NHE	non-hemolytic enterotoxin
NMKL	Nordic Committee on Food Analysis
PCR	Polymerase chain reaction
PEMBA	Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplification of polymorphic DNA
rep-PCR	Repetitive Polymerase Chain Reaction
RM	Ringgit Malaysia
Rpm	Revolution per minute
RTE	Ready-to-eat
SLST	Single locus sequence typing

U	Unit
UV	Ultra violet
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TBS	Tryptone soy broth
USDA	United States Department of Agriculture
VNTR	Variable number tandem repeat
WHO	World Health Organization

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In Sarawak, sago processing industries is a well-established industry that contribute to Sarawak export revenue (Karim *et al.*, 2008). Sago palm is the plant that has high potential agricultural industry and is grown commercially in Southern East Asia countries (Singhal *et al.*, 2008). According to Adeni *et al.* (2009), sago is very important food product for over a million peoples as their primary dietary starch source. Starch is one of the most abundant plant products in the world. It is a major source of energy in human daily food. Every year, the consumption of the sago starch by the global citizen is estimated to be between 200,000 to 300,000 tons (Bujang and Muniandy, 2004). The export of sago product from Sarawak in 2013 was estimated to be 50,000 tonne which procuring income approximately RM 81 millions (Department of Agriculture Sarawak, 2013). Usually, sago starch is used for various applications in Malaysia such as glucose, monosodium glutamate and noodles (Bujang, 2008).

1.1.1 Problem Statement

In food processing industries, some of the preparation, processing and storage procedures were exposed to the risk of bacterial contamination. It might harbor a great number of microorganisms that may cause the food products to spoil and represents a direct health hazards to consumers (Lesley *et al.*, 2013). Most of the incidence of diarrhoea in developing countries are caused by foodborne and waterborne pathogens (WHO, 2008). Diarrhoeal diseases are included in 20 leading causes of death in the world in 2014 (WHO, 2014). Unhygienic food handling and preparation and inadequate cooking which may lead to food

poisoning due to the presence of bacteria. (Sandra *et al*., 2012). Until March 2015, 143 cases of food poisoning were reported in Sarawak (Sarawak EPID Health News, 2015). There are a few food poisoning bacteria reported to be commonly found in food and its processing which include *Salmonella* spp., *B. cereus, Escherichia coli* and *Listeria* species (Greenhill *et al.,* 2007).

B. cereus is chosen as potential hazard due to its ubiquitous nature and its preference to live in soil and starchy food. The presence of the *B. cereus* in food processing industries cause problems by reducing the quality of the products (Lesley *et al.,* 2013; Sandra *et al.,* 2012), and affecting people's health after eating the contaminated foods (Ghelardi *et al.,* 2002). *B. cereus* was first discovered by Frankland *et al*. (1887) in the air of the cowshed. *B. cereus* affects foods processing industry both by reducing the quality of the products (Lesley *et al.,* 2013; Sandra *et al.,* 2012) and by endangering people's health upon eating contaminated foods (Ghelardi *et al.,* 2002).

However, to date, there is limited study of the occurrence of microorganisms including *B. cereus* in sago-associated products has been conducted (Greenhill *et al.,* 2007). To the best of our knowledge, there has been no study on the prevalence of *B. cereus* in sago processing has been reported in Sarawak. Hence, in our study we isolated and identified *B. cereus* from sago processing and determine the prevalence of the *B. cereus.* There are several international standard protocol commonly used for isolation and identification of *B. cereus* which include the US Food and Drug Administration (FDA), the Association of Official Analytical Chemists (AOAC), the European Committee for Standardisation (CEN, EN), the International Organization for Standardization (ISO), the Netherlands Government Food Inspection Services (NGFIS), the Nordic Committee on Food Analysis (NMKL) and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS). In our study, we applied

FDA method which involve the sample enrichment by using tryptone soy broth and direct plating on MYP agar (Lesley *et al.*, 2013).

B. cereus is further identified and confirmed by PCR assay. Further confirmation and identification by PCR assay were required as conventional detecting method often give false-positive/false-negative results. Hence PCR assay can be applied as it is reliable, specific, sensitive and reproducible method. In the present study, we detect *B. cereus* by targeting haemolysin gene as described by Mutashar *et al.* (2015), Fukushima *et al.* (2003) and Wang *et al*. (1997). The raw material quality and the processing environment of food products can affect the final products quality (Oh *et al.*, 2012). As contamination and/or cross-contamination were the major concern in food processing, hence we decided to conduct molecular typing of *B. cereus* by using rep-PCR method in order to determine the contamination pattern in the sago processing mills. It is important to understand the routes of contamination of *B. cereus* in sago processing to prevent the contamination of the final products (Oh *et al.*, 2012).

B. cereus is an opportunistic pathogen that can cause severe foodborne illness. There were also several studies on the resistance of *B. cereus* toward antibiotics had been reported (Yim *et al.*, 2015; Lee *et al*., 2012; Park *et al.*, 2009; Rosenquist *et al.*, 2005 and Andrews *et al.*, 2002). This showed it is necessary to investigate the *B. cereus* antibiotic resistance and susceptibility. Antibiotics have been widely used in agriculture, veterinary and medicine. Prolonged usage and exposure towards commonly used antibiotics including penicillin, ampicillin, norfloxazin, imipenem, tetracycline, chloramphenicol, quinolones, aminoglycoside, spectinomycin, cephalosporin, nitrofuran, nitromidazole, sulfonamide, and trimethoprim are able to promote the emergence of resistance in bacteria. It is important to monitor antibiotic susceptibility of *B. cereus* in food processing to ensure and monitor the emergence and spread of bacterial resistance to antimicrobial agents. However, there is no data of antibiotic resistance

of bacteria associated with sago processing mills. In this study, the antibiotic susceptibility of *B. cereus* in the sago was examined by disk diffusion method (Park *et al.,* 2009). The findings of this study provided baseline data on *B. cereus* contamination for future risk assessment work and establishment of food safety standards in sago processing in Sarawak.

1.1.2 Objectives

The main goal of this study was to detect, quantify and characterize *B. cereus* in sago processing in Sarawak, Malaysia. The specific objectives of this study were to:

a. Detect *B. cereus* in sago processing by using Polymerase Chain Reaction.
b. Determine the contamination level of *B. cereus* in sago processing by enumerating *B. cereus* using standard plate count method.
c. Determine the antibiotic resistance profiles of *B. cereus* isolated from sago processing.
d. Characterise *B. cereus* in sago processing by using Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC- PCR) and Pulsed-field Gel Electrophoresis (PFGE).

1.2 Literature review

1.2.1 Sago palm

Sago palm (Figure 1.1) or *Metroxylon sagu* belongs to the genus *Metroxylon* of Palmae family (Greenhill *et al.,* 2007). *Metroxylon sagu* is derived from the term 'metra' which means pith or parenchyma and the term 'xylon' means xylem. Many scientists considered it as the starch crop of the 21st century (Singhal *et al.,* 2008). It is due to its ability to store and yield more starch in its stem than that of any other starch crop (Onsa *et al.,* 2004). *Metroxylon sago* is able to survive swamp area, acidic peat soils and in high saline area (Ehara *et al.,* 2000). It needs little management and able to live in marginal agricultural land (Purwanto *et al.,* 2002).

The plant is believed to have originated from Papua New Guinea and/or Moluccas where Papua New Guinea considered as the center of the diversity of sago palm (Toyoda, 2008). In Malaysia, Sarawak is the largest area for the *Metroxylon sagu* plantation which is approximately 55500 hectares in 2012 (Department of Agriculture Sarawak, 2013). Seventy-five percent of Sarawak sago planting area are found in Mukah (Bujang, 2010).

Figure 1.1: Sago plantation in Mukah, Sarawak (Bujang, 2008).

1.2.2 Sago processing

Sago was processed commercially using the conventional and modern method in Sarawak (Shin and Collins, 2015). Conventional method and modern method had been described by Shin and Collins (2015) and Bujang (2008), respectively. Modern method usually being practiced in sago processing mills. In the modern method, mature sago trunk was selected and cut. After sago trunk is cut, it will be further cut into smaller one meter logs (Figure 1.2). The smaller logs of sago trunks will be transported to the sago processing mill by using lorries

or tied to form rafts so that they can be transported via river to the mill (Figure 1.3). Then, the bark of the sago trunk will be debarked manually or using automated debarking machine (Figure 1.4). The debarked logs are mashed using a rasper, followed by a hammer mill and then water is added to form starch slurry (Figure 1.5). Then, the starch slurry is allowed to settle down in the tank, dry and finally form sago flour that are ready for packaging (Figure 1.6). Figure 1.7 shows the schematic flow diagram of the sago processing.

Figure 1.2: Sago trunk was selected before cut into smaller 1 meter log.

Figure 1.3: Sago logs were transported to the sago processing mills via river.

Figure 1.4: Sago logs were debarked automatically by using automated machine (left) or manually by human (right).

Figure 1.5: Debarked sago logs were mashed into a rasper (left) and water is added to form starch slurry (right).

Figure 1.6: Starch slurry is allowed to settle down in the tank, dry and finally form sago flour that are ready for packaging.

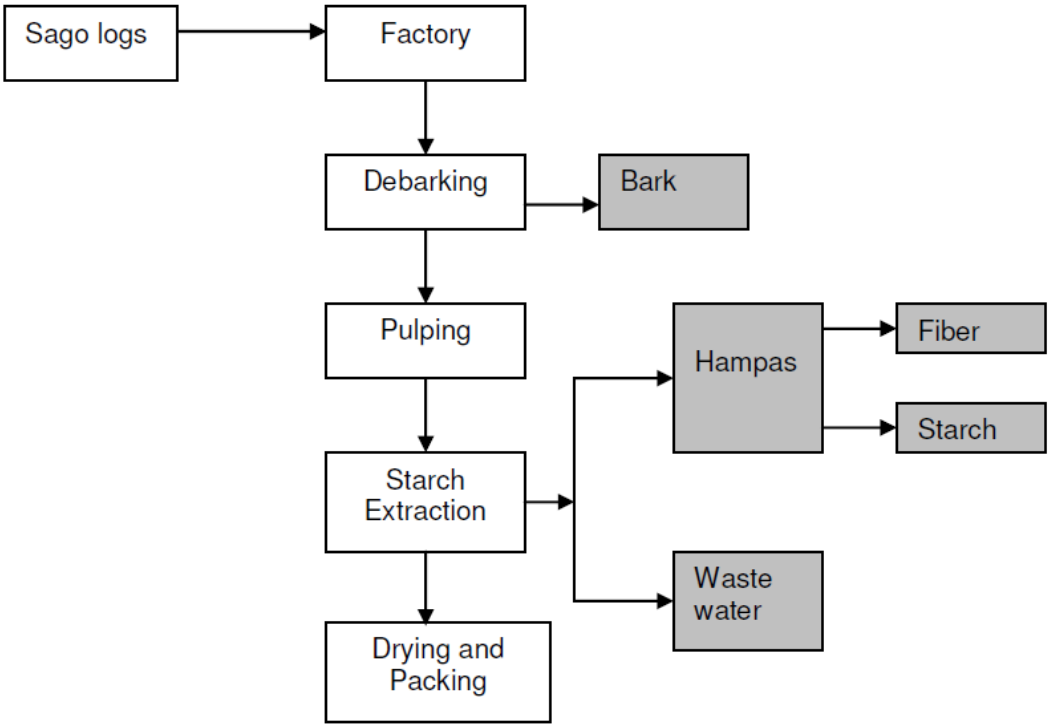

Figure 1.7: Schematic flow diagram of the sago processing (Bujang, 2008).

1.2.3 Morphology and characteristics of *B. cereus*

B. cereus is a Gram-positive, rod-shaped foodborne pathogen that can cause gastrointestinal disease such as food poisoning (Sandra *et al.,* 2012; Kotiranta *et al.,* 2000). *B. cereus* is commonly found in soil and is able to live in it because it does not have complex nutrient requirements (Kotiranta *et al.,* 2000). *B. cereus* can be isolated from a wide range of food such as milk, dairy, ready-to-eat cereals, rice or starchy food (Lesley *et al.,* 2013; Greenhill, 2010; Kontiranta, 2000). The bacterium is able to live within the temperature of 4°C to 55°C with the optimal temperature of 28–37°C. *B. cereus* is able to grow in pH between 4.9 to 9.3 and able to survive 7.5% salt concentration (Lampel *et al.* 2012). The bacterium produces spore which can endure heat, dehydration and other physical stress (Stenfors, 2008) and can

resist pasteurization process (Acai *et al.,* 2014). *B. cereus* can cause two different type of food-associated illness such as emetic and diarrheal type (Granum, 1997). Emetic type food associated illness causes by small-molecular weight cyclic toxin, cereulide whereas the diarrheal type foodborne illness causes by the enterotoxins (Rosenquist *et al.,* 2005). Cereulide is formed in food while the enterotoxins are formed in the intestine after the consumption of *B. cereus* (Ehling-schulz *et al.,* 2004). Generally, foods containing > 10^4 *B. cereus*/g are not considered as safe.

1.2.4 Toxin genes of *B. cereus*

B. cereus has a few toxic genes namely haemolytic BL and non-haemolytic BL (Banerjee *et al.,* 2011). Haemolytic BL responsible for the enterotoxigenicity such as diarrhoeal food poisoning syndrome and necrotizing infections such as endophthalmitis (Reis *et al.,* 2013). Haemolysin BL consisted of three different protein antigen which is the binding site named B and lytic component named L_1 and L_2 (Kim *et al.,* 2008) and are encoded with gene *hblA*, *hblC*, and *hblD* respectively (Reis *et al.,* 2013). The non-haemolytic enterotoxin (NHE) complex consist of protein A, B and C with molecular mass 39, 45, and 105 kDa respectively (Owusu-kwarteng *et al.,* 2017; Kim *et al.,* 2008 and Lund *et al.,* 2000). This complex are cytotoxic in low concentration and responsible for the cytotoxicity of *B. cereus*. The corresponding encoding genes are *nheA*, *nheB* and *nheC* for protein A, B and C respectively. (Kim *et al.,* 2010). Kim *et al.* (2010) detected *B. cereus* in all isolates using non-haemolytic virulence gene, *nheA, nheB* and *nheC.* Hendrikson (2001) detected enterotoxic *B. cereus* by targeting *hblA, hblC,* and *hblD* genes of the HBL complex, and the *nheA, nheB* and *nheC* genes of the NHE complex in *B. cereus.*

1.2.5 Outbreaks of *B. cereus*

An outbreak of *Bacillus peptonificons* which was later known as *B. cereus* involving 300 patients and staff in a hospital in 1906 was among the first outbreak reported (Evans, 2013; Doyle, 1989). They developed acute gastroenteritis symptoms shortly after eating their dinner. Foodborne Disease Outbreak Surveillance System in United States recorded that seven and five cases of *B. cereus* outbreaks occurred in 2010 and 2009 respectively (CDC, 2013). In addition, Mead *et al*. (1999) reported that approximately 27,360 cases of food-borne illness reported in United States in year 1997 caused by *B. cereus*. (Mead *et al.,* 1999). Five hundred and seventy one cases of *B. cereus* foodborne poisoning were also recording in US between 1998 and 2002 (Michael *et al.,* 2006). In Korea, 5.5% (18 cases) of the total outbreaks in 2009 were caused by *B. cereus* (Chang *et al,*. 2011).

In August 2004, there was another outbreak of *B. cereus* whereby 90 cadets from University Military Program in Georgia were involved (CDC, 2005). Another case reported in August 2003, a fatal family outbreak where five children became ill after eating pasta salad contaminated with *B. cereus* (Dierick *et al.,* 2005). Recently in New York, Mighty Taco recalled refried beans from all of its restaurant because people became ill and showed nausea and vomiting symptoms after eating food contaminated with *B. cereus* (Food Safety News, 2016). In Malaysia, a food poisoning caused by *B. cereus* occurred in one of the school hostel in Klang affecting 114 female. They developed abdominal pain, nausea, vomiting and giddiness after consumed fried noodle served by school dining hall (Rampal *et al*., 1984). However, there is minimal data on *B. cereus* foodborne illness outbreak in Malaysia due to under reporting by state public health laboratories (Sandra *et al.,* 2012).

1.2.6 Prevalence of *B. cereus* in food and food processing plant

There are limited studies reported on the prevalence of *B. cereus* in sago and/or sago processing plant. Greenhill *et al.* (2007) in their research reported 32 out of the 43 sago samples were detected with *B. cereus*. The high prevalence of *B. cereus* were due to extraction and processing techniques in sago production. In another study, Greenhill *et al.* (2009) reported that trunk growth and storage on contaminated soil and water, poor hygienic practices among handlers and workers, migratory animals and contaminated and improper washed and sanitized equipment were the potential hazards along the sago processing.

However, due to its ubiquitous nature, *B. cereus* had been isolated from various types of food and their processing plant. Microorganism can contaminate food and crop along the food production chain, including during pre-harvest where crops were planted or animals were raised, harvest and post-harvest consisting of food processing, distribution and marketing, storage, preparation and serving (Akanele *et al.,* 2016). Kim *et al*. (2016) in their study revealed that salad samples from raw materials, washing steps, and final products exhibit 27.3 to 30.8 % prevalence of *B. cereus* while the occurrence of environmental samples was very low (16.7%). Contamination have might occurred in raw materials and cross-contaminated with equipment along the processing line. The prevalence of *B. cereus* also has been studied in ready-to-eat (RTE) vegetables in Southern Korea (Chon *et al.*, 2015). Their study showed that *B. cereus* present in 48% (70/145) of the retail vegetable salad and sprout samples tested. In Malaysia, the prevalence of *B. cereus* in food had been conducted by Sawei *et al.* (2016) in indigenous paddy, Lesley *et al.* (2013) in RTE cereals and Sandra *et al*. (2012) in various types of rice. Sawei *et al*. (2016) reported 93.33% (84.90) paddy samples from local cultivators were positive of *B. cereus*. *B. cereus* can contaminate paddy during growth, harvesting, milling and other agricultural operations. The prevalence of *B. cereus* in RTE cereals in Sarawak also had been

reported (Lesley *et al.,* 2013). In their study, *B. cereus* contaminated four of the 30 RTE cereals food samples. Even though *B. cereus* mostly live in soil, it can cross-contaminate in raw cereals plant. It also can cross-contaminate the RTE cereals in various way including from the workers, packaging process and the equipment that present along the processing line. The prevalence of cooked rice samples such as *nasi lemak, nasi briyani, nasi ayam* and *nasi putih* were also studied (Sandra *et al.,* 2012). Out of 115 cooked rice samples studied, 84 samples were detected with *B. cereus*. There are a lot of factors that affects the prevalence of *B. cereus* in food products and its processing. However, strict processing conditions, appropriate management practices and strict continuous surveillance from the authorities can minimize the risk of the contamination.

1.2.7 Isolation and enumeration of *B. cereus* using conventional method

There are several conventional methods that had been published in international standards to isolate and identify *B. cereus* such as the standard of the United States Food and Drug Administration (FDA), the International Organization for Standardization (ISO) and the US Department of Agriculture–Food Safety and Inspection Service (USDA–FSIS). According to FDA standard, *B. cereus* was enriched in trypticase soy-polymyxin broth before being streaked on selective agar such as Bacara and Mannitol- Egg-Yolk-Polymyxin (MYP) agar. Sandra *et al.* (2012) isolated 100% of *B. cereus* in *nasi ayam*, 76.2% in *nasi putih*, 70.4% in *nasi lemak* and 50% in *nasi beriyani*, by using this method. They suggested that common culturing method should be used in combination with PCR-based detection method as conventional culturing method alone can resulted in false negative as reported in their study. Valero *et al.* (2002) applied ISO method in their study to isolate *B. cereus* in vegetables. In their study, 10 g of samples were enriched in 90 mL of peptone water before spreading one milliliter of suspension on the selective agar, *B. cereus* agar base with polymyxin B and egg yolk sterile

solution. Thirty-six samples were detected with *B. cereus*. For USDA-FSIS method, samples were enriched with Butterfield's Phosphate Diluent (BPD) prior streaking on MYP agar. In the study by Chon *et al.* (2015), they studied the prevalence of *B. cereus* in RTE vegetables by using the method described by USDA-FSIS. In the study, they found that *B. cereus* were detected in 48% (45 out of 145) of vegetable salad and sprout.

There are various methods of bacterial enumeration described such as Most Probable Number (MPN), standard plate count method, Modified Semisolid Rappaport-Vassiliades (MSRV) and direct counting to enumerate microorganisms (Malorny *et al.*, 2008). Enumeration of bacteria is important in order to determine the concentration of bacteria in the samples so that the contamination level can be assessed (FDA, 2012). Among all the method, standard plate count are frequently used to enumerate the bacteria in the samples. Despite its laborious and time-consuming method, it still serves as one of the most common methods applied to assess the level of contamination in food. In standard plate count method, bacterial enrichment broth with appropriate dilution are spread on selective agar before subjected to incubation in certain temperature and time (Food Standards Australia New Zealand, 2001). After incubation, bacterial colonies can be counted and colony-forming units (CFU) for the particular bacteria can be calculated. Greenhill *et al.* (2007) had enumerated *B. cereus* in sago starch in Papua New Guinea by plating the tryptone soy polymyxin broth on PEMBA agar. They revealed that the concentration of *B. cereus* in 58% (25/43) samples were less than 10^2 CFU/g whereas 23% (10/43) of samples were between 10^2 and 10^3 CFU/g.

1.2.8 Isolation of *B. cereus* using polymerase chain reaction (PCR)

Sometimes the isolation and enumeration of *B. cereus* by using conventional method showed false positive result. Hence, polymerase chain reaction was carried out to further

confirm the result. Polymerase chain reaction (PCR) is a molecular technique to amplify the copy number of target gene of interest (Pasic *et al.*, 2014). This molecular technique was firstly introduced by Dr. Kary Mullis and colleagues in 1983. In 1983, Dr. Kary Mullis was awarded the Nobel Price in Chemistry (Julin, 2012) and his achievements revolutionized the field of molecular biology. PCR assay is a simple and rapid way to amplify specific copy of interest nucleic acid and have been widely used in clinical and food quality control analysis. PCR is a chain reaction that require DNA template, DNA polymerase, a set of forward and reverse primers, deoxynucleotide triphosphates (dNTP) and magnesium ion. DNA polymerase is a heat-stable enzyme that synthesizes new strand from the targeted gene where as dNTPs are the bases T, A, G and C that are essential for the building of multiple new copies of DNA strands (Agne *et al.*, 2009). Basically, PCR cycles has three basic steps of amplification as summarized in Figure 1.8 which consist of denaturation, annealing and extension (Pasic *et al.*, 2014). In denaturation process, heat is applied to break the bond between two strand of DNA and separate the double-stranded DNA into single-stranded DNA. Next, in annealing process, the primers bind to the specific and targeted DNA sequence. DNA polymerase synthesizes two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA then the new strands undergo further duplication. The annealing phase usually occurs at a lower temperature, from 50-60°C to allow the primers to hybridize to their respective complementary template strands. Extension occurs at the end of the annealing phase to create a complementary copy strand of DNA. The extension phase occurs at approximately 72°C to fill in the protruding ends of newly synthesized PCR products (Garibyan and Avashia, 2013).

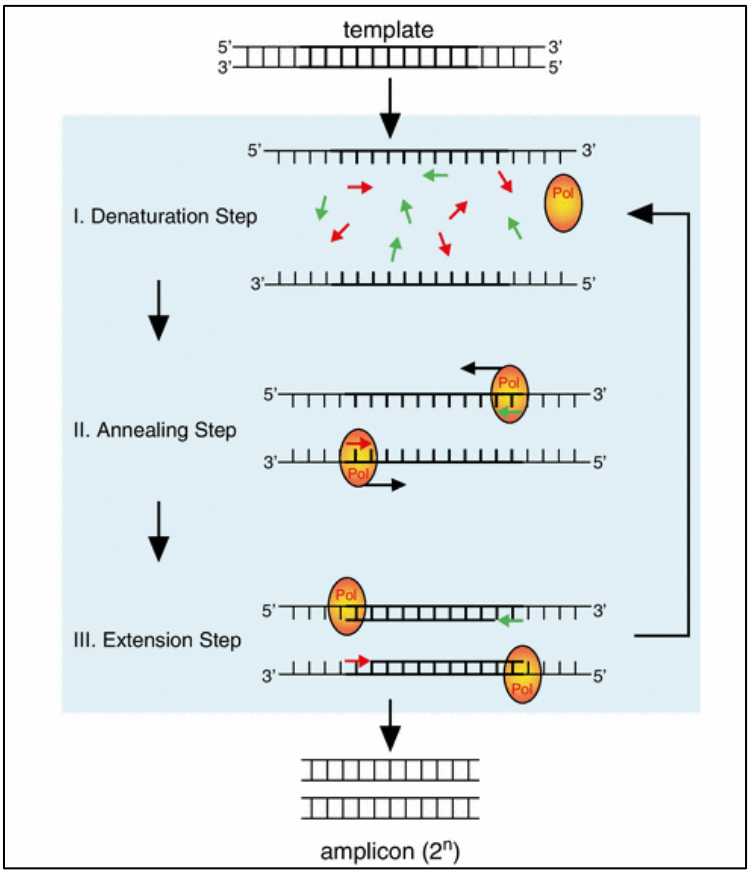

Figure 1.8: Overview of PCR amplification process (Pasic *et al.*, 2014).

Wang *et al.* (1997) developed a universal protocol for PCR detection of 13 species of foodborne pathogens in foods including *B. cereus*. In their study, the detection of *B. cereus* were by targeting haemolysin gene with the primers BC-1, CTGTAGCGAATCGTACGTATC and BC-2, TACTGCTCCAGCCACATTAC with the product size 185 bp (Wang *et al.*, 1997). Fukushima *et al*. (2003) and Mutashar *et al.* (2015) in their research also detected the presence of *B. cereus* in stools and cheese respectively by PCR assay targeting haemolysin gene of *B. cereus*. Haemolysin gene of *B. cereus* responsible for the enterotoxigenicity such as diarrhoeal food poisoning syndrome and necrotizing infections such as endophthalmitis (Reis *et al.*, 2013). By using PCR assay, Sandra *et al*. (2012) detected *B. cereus* in 73.04% out of 115 rice samples in Malaysia. Park *et al.* (2009) detected the presence of *B. cereus* in rice and cereals in Korea.

Among 293 rice and cereal samples tested, 73 (25%) samples consist of brown rice, glutinous rice, barley and Job's tears.

The PCR products obtained from PCR is then subjected to agarose gel electrophoresis (AGE) in order the separate the fragments of PCR product. AGE can separate DNA fragments sized between 100 bp to 25 kb effectively and efficiently (Lee *et al.,* 2012). Before AGE was introduced, DNA was separated by using sucrose density gradient centrifugation, which only provides an approximation of size (Das & Dash, 2015). After the DNA is loaded into pre-cast well in the gel and current is applied, DNA molecules are separated by size. The rate of migration of a DNA molecule through a gel is influenced by size of DNA molecule, agarose concentration, DNA conformation, voltage applied, presence of ethidium bromide, type of agarose and electrophoresis buffer. After separation, the DNA molecules can be visualized under UV light after staining with an appropriate dye (Lee *et al.,* 2012). PCR is one of the reliable technique to detect the microorganisms in food in a rapid way. It has higher sensitivity and enhance the detection of pathogenic bacteria (Lampel *et al.,* 2000).

1.2.9 Molecular typing of *B. cereus*

Molecular typing are essential epidemiological tools in surveillance of bacterial pathogens in infection prevention and control. They are used to identify different bacterial isolates within the same species (Lin *et al.,* 2014). There are few types of molecular typing methods which include pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), repetitive element PCR (rep-PCR), variable-number tandem repeat (VNTR), single locus sequence typing (SLST), multi-locus sequence typing (MLST), comparative genomic hybridization, optical mapping and whole genome sequencing. Several factors require to be considered such as

discriminatory power, reproducibility and typeability, as well as the biological basis for grouping similar strains, cost, and logistics; in order to select a genotyping strategy. It is due to they have their specific advantages and disadvantages. (Tenover *et al.,* 2012). Genotyping of *B. cereus* isolates were able to detect the causes, sources and microorganism contamination route during processing (Chen *et al.,* 2004).

There are few low cost, fast and reliable PCR-based typing techniques can be done to identify the genetic relatedness such as enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). ERIC sequences are usually 124-127 bp long, highly conserved central inverted repeat and located at non-coding transcribed regions of the chromosome of various enterobacteria (Fakruddin *et al.,* 2013 and Waturangi *et al.,* 2012). ERIC sequences were first discovered in *Salmonella typhi* and *E. coli* and make up approximately 1% of the bacterial genome. ERIC-PCR reveals a profile of DNA fragments of different sizes based, in principle, on the genomic locations of specific repetitive sequences.

The genetic relatedness also can be further confirmed by pulsed-field gel electrophoresis (PFGE). It is a technique where the large DNA fragments were restricted by using restriction enzyme and these DNA fragments were separated on gel matrix with an electric field that changes direction periodically. PFGE can be used to analyze large DNA fragments of bacterial chromosome. PFGE is a powerful tool that has high discriminatory value in characterizing different strain at DNA level (Basim, 2001). However, PFGE analysis is laborious and time-consuming, hence the use of ERIC-PCR as a complementary tool for molecular typing (Merzougui *et al.,* 2013) is more preferable. In previous work by Oh *et al.* (2012), genotyping of *B. cereus* in red pepper powder were carried out by using rep-PCR method. It was revealed that half of the samples were clustered together and more than two isolates had the similarity more than 95%. High genetic similarities were detected in the samples from raw materials,

crude milling, and milling. It revealed that these isolates may have originated from the same clone. Merzougui *et al.* (2013) in their study also demonstrate the genotyping of *B. cereus* in several food samples in Morocco such as dairy products, spices, milk and rice salads by using ERIC-PCR and PFGE. In their study, all the isolates showed genetic diversity in the samples. They also compare the strengths and weaknesses of each method whereby they suggested that despite of laborious and time-consuming process of PFGE, this genotyping method have higher discriminatory power compared to ERIC-PCR. Hence, this is the reason why PFGE is a gold standard for DNA fingerprinting. ERIC-PCR on the other hand is cheaper, faster and more sensitive for genotyping. Kim *et al.* (2016) studied the contamination pattern and genotyping of *B. cereus* in fresh-cut vegetables in four manufacturing factories in Korea. High genetic similarities were exhibited in the manufacturing factories. This indicates the same *B. cereus* strain had been circulated throughout the processing line and it may be originated from the raw materials.

1.2.10 Antibiotic susceptibilities test of *B. cereus*

Antibiotics are chemical substance produced in nature (Tortora *et al.,* 2007) that are able to inhibit and destroy the growth of bacteria. In medical field, antibiotics were used to treat bacterial infection (WHO, 2016). Antibiotics are one of the most significant success of chemotherapy in the history of medicine where by a lot of lives are able to be saved and a lot of infectious diseases can be controlled (Aminov, 2010). The first antibiotic, penicillin was accidentally discovered by Alexander Fleming in 1928. He observed the growth of *Staphylococcus aureus* were inhibited by a colony of *Penicillium notatum* that contaminated his culture (Byarugaba, 2010). However, years after the discovery of antibiotics, there were still organisms that are able to survive against antibiotics which is known today as antibiotic resistance (Byarugaba, 2010). Fleming was among the first who was aware of the potential

resistance of penicillin if the antibiotic is improperly used during treatment (Aminov, 2010). Improper uses of antibiotics in medicine often lead to antibiotic resistance where the bacteria are evolving into more competent bacteria and manage to survive environmental stress including antibiotic. Antibiotic resistance becomes a threat to developing countries and is growing in alarming pace (Sosa, 2010). According to Gelband (2015), antibiotic resistance is due to the usage of antibiotic. The volume of antibiotics used are directly proportional to the chances that antibiotic resistant populations of bacteria will prevail in the contest for survival of the fittest at the bacterial level. This poses great risk towards the public health because the antibiotic resistant gene are able to be transferred to humans via the food chain (Fallah *et al.,* 2013).

B. cereus had been reported to be highly resistant to β-lactam antibiotics such as ampicillin, penicillin, cefepime and oxacillin. Yim *et al.* (2015) reported that *B. cereus* isolated from soy products were 100% resistant towards ampicillin, cefepime, oxacillin and penicillin. Lee *et al*. (2012) also revealed the same trend where all the isolates were highly resistant towards the same antibiotics. The isolates were highly susceptible to chloramphenicol, gentamicin, imipenem, sulfamethoxazole, and tetracycline. Park *et al.* (2009), Rosenquist *et al.* (2005) and Andrews (2002) also produced the similar trend of result. Eighty years of increased usage of antibiotic have created multiple antibiotic resistant pathogenic bacteria.

1.2.11 Multiple antibiotic resistance (MAR) index

Multiple antibiotic resistance (MAR) indexing is a reliable and inexpensive method to track the sources of bacteria. MAR indexing was first introduced by Krumperman *et al.* (1983). MAR index is calculated as the ratio of number of resistant antibiotics to which organism is resistant to total number of antibiotics to which organism is exposed. In their study, a total of

9000 *E. coli* isolates were randomly obtained from a wide range of sources such as human, domestic animals, farm animals, wild animals, rural or urban vectors animals, orchard soil and sewage water. These samples were easily defined as samples that were highly exposed to antibiotics and vice versa. For example, domestic animals such as grazing cattle and sheep, wild animals, rural vector animals and orchard soil were samples that represent the sources where antibiotics were rarely used. The MAR index for these samples were less than 0.2. However, on the other hand, farm animals, urban vectors animals, sewage water and human anal swab were represent the samples that were highly exposed to antibiotics. Hence, MAR index for these samples were more than 0.2. Therefore, Krumperman *et al.* came with the cut off value of 0.2 which MAR index that are more than 0.20 indicates that the organism was originated from sources of contamination where antibiotics were often used (Krumperman, 1983).

MAR indexing were also applied in a lot of studies later on. Previous study on the detection and determination of MAR index of bacteria isolated from aquaculture farm showed MAR index ranging from 0.03 to 0.43 (Liyanage and Manage, 2017). Hora and Ali (2017) examined the antibiotic resistance of *E. coli* isolated from human clinical samples in India. The MAR index of the isolated were between 0.18 and 0.89. Silvester *et al.* (2015) also conducted a study on the antibiotic resistance of *Vibrio parahaemolyticus* from a tropical estuary and adjoining traditional prawn farm along the southwest coast of India. The MAR index of the isolates from the Cochin estuary ranged from 0.312 to 0.75 and that from the shrimp farm ranged from 0.1875 to 0.5. In Malaysia, Sandra *et al.* (2012) conducted the antibiotic susceptibility test of *B. cereus* isolated in cooked rice and beef patties. The isolates shows multi-resistance towards 8 antibiotics tested. 42.4% of the isolates showed MAR index of 0.25 whereas 54.3% of the isolates showed MAR of 0.36. The other study on bacteria isolated from aquaculture in Sarawak, MAR index of the bacterial isolate were ranging from 0.167 to 0.333

(Samuel *et al.*, 2011). These indicated that there is a seriously misuse of antimicrobials and poses a real threat to public health.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample collection

A total of 120 samples of sago effluent, starch slurry, sago flour and sago *hampas* were collected from two sago processing factories located at Pusa and Mukah Sarawak (Figure 2.1) from January 2016 until December 2016. Ten samples were collected from each step of processing which includes debarking, pulping, starch extraction, drying and packaging. Approximately 50 mL of liquid samples such as starch slurry and sago effluent were collected using sterile 50 mL screw-capped Falcon's tube. In addition, approximately 100 g of sago flour and sago *hampas* were also collected using sterile plastic bag by using sterile spade. Collected samples were transported to the lab by using ice box containing ice within 24 hours.

Figure 2.1: Location of sago processing A (Pusa) and sago processing B (Mukah) (Source: Google Maps).

2.2 Sample processing

Ten milliliters of starch slurry and sago effluent whereas 10 g of sago flour and sago hampas were aseptically placed in the stomacher bag and were homogenized in 90 mL Tryptic Soy Broth (Appendix 1.1). Then, 1 mL of homogenized samples were pipetted into 9 mL of Tryptic Soy Broth and incubated for 24 hours at 37ºC with agitation of 120 rpm.

2.3 Isolation of *B. cereus*

After 24 hours of incubation, serial dilution were performed until 10^{-6} in Phosphate Buffered Saline (Appendix 1.4). One milliliter of overnight culture was pipetted into 9 mL of saline solution and labeled as dilution 10^{-1}. One hundred microliters of each dilution were plated onto Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar (Appendix 1.2) using spread plate technique. Duplicates were made for each dilution and the polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar (PEMBA) were incubated at 37ºC for 24 hours. After incubation, plates were examined for the presence of peacock blue colonies on peacock blue medium. The colonies on the polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar (PEMBA) were enumerated using plate count method to calculate the colony forming unit (CFU) per milliliter of the samples. The total bacteria (CFU/mL) were calculated according to the formula from previous study (Kim *et al.*, 2016) as shown below:

$$CFU/mL = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of culture plated}}$$

Next, seven to ten single colonies were randomly chosen from dilution containing 30 to 300 single colonies and were streaked on new *B. cereus* selective agar to obtain pure colony. Pure

colonies of each isolate were kept in slant agar (Appendix 1.4) and 50% glycerol stock (Appendix 1.5) for long term storage.

2.4 Genomic DNA extraction

Extraction of bacterial genomic DNA was conducted using Bacterial Genomic DNA Isolation Kit by Norgen Biotek Corp. The bacterial culture were made by inoculating pure colonies of *Bacillus cereus* into 9 mL of tryptic soy broth and incubated for 24 hours at 37 °C with agitation of 120 rpm.

One milliliter of bacterial culture was transferred to a sterile 1.5 mL microcentrifuge tubes and was centrifuged at 14,000 rpm for 30 seconds to pellet the cells. The supernatant was poured carefully so as not to disturb or dislodge the cell pellet. Then, 250 µL of resuspension solution A was added to the cell pellet. The cells were resuspended by gentle vortexing. Two hundred fifty microliter of Lysis Buffer P and 12 µL of Proteinase K were added to the cell suspension. The mixture was mixed well by gentle vortexing and was incubated at 55^{o}C for 30 minutes. After the incubation, 500 µL of Solution BX was added to the lysate and mixed well with gentle vortexing to ensure homogeneous mixture is obtained.

A spin column is obtained and assembled with a collection tune. Seven hundred fifty microliter of the mixture was added to the spin column assemble. The column was capped and centrifuged for 1 minute at 8000 rpm. After the centrifugation, the flowthrough was discarded and the spin column was reassembled with its collection tube. The rest of the mixture was added and was centrifuged again for 1 minute at 8000 rpm. After the centrifugation, the flowthrough was discarded again and the spin column was reassembled with its collection tube.

Five hundred microliter of Wash Solution A was applied to the column. The mixture was centrifuged at 14,000 rpm for 1 minute. The flowthrough was discarded the spin column

was reassembled with its collection tube. After that, the column was washed by adding another 500 µL Wash Solution A for the second time and was centrifuged at 14,000 rpm for 1 minute. The column was spinned for 2 minutes in order to thoroughly dry the column before the collection tube is discarded. The spin column was assembled with a provided 1.7 mL elution tube. 200 µL of Elution Buffer B was added to the center of the column bed. Next, it was centrifuged for 1 minute at 6000 rpm. The portion of Elution Buffer B passed through the column allow the hydration of the DNA to occur. The purified genomic DNA was restored at -20^{o}C for later study.

2.5 Detection of haemolysin (*hly*) gene by using PCR

Specific PCR was conducted according to Wang *et al.* (1997). PCR was performed to target on haemolysin of *B. cereus*. The sequence of the primers with the gene products are described in Table 2.1.

Table 2.1: The primers used

Gene Target	Sequence	Size (bp)	Reference
haemolysin gene (*hly*)	F 5'-CTGTAGCGAATCGTACGTATC-3' R 3'-TACTGCTCCAGCCACATTAC-5'	185 bp	Wang *et al.*, 1997

Amplification of the DNA was conducted in a total volume of 25 µL as described by (Sandra *et al*., 2012) with modification. The reaction mixture consisted of 5X *Taq* Green buffer, 25 mM of $MgCl_2$, 25 mM of dNTP's premixed, 10 mM of each primers and 0.5 unit of *Taq* Polymerase DNA. The concentration and volume per reaction of the PCR mixtures is listed in Table 2.2. The amplification was amplified in thermal cycler according to the cycling conditions as listed in Table 2.3.

Table 2.2: The PCR components

PCR components	Concentration	Volume per reaction (µL)
Taq green buffer	5X	5.00
$MgCl_2$	25 mM	5.00
Deoxyribonucleotide phosphate (dNTPs)	25 mM	2.00
Primer haemolysin– F	10 µM	0.50
Primer haemolysin– R	10 µM	1.00
DNA template	20 – 30 ng	1.00
Taq DNA polymerase	5 U	8.75
Sterile distilled water (dH_2O)		10.25
Total		25

Table 2.3: Cycling conditions for haemolysin gene (*hly)*

Condition	Temperature (ºC)	Time (minute)	Number of cycles
Initial denaturation	94	10.0	1
Denaturation	94	0.5	35
Annealing	63	1.0	35
Extension	72	1.0	35
Final extension	72	10.0	1

Five microliter of PCR product together were subjected to electrophoresis on a 2.0% (w/v) agarose gel in 1X TBE buffer at 90 V for 1 hour 45 minutes. The 100 bp DNA marker (Vivantis) were included. Then, the agarose gel was pre-stained with ethidium bromide and visualized with the aid of UV transilluminator (Maestrogen).

2.6 Antibiotic susceptibility test (AST)

The bacteria isolates were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method from previous work by Kim *et al.* (2015) which was according to the Clinical Laboratory Standards Institute. Few single colonies were picked and inoculated into 5 mL phosphate buffered saline (PBS). The turbidity of the bacterial culture was compared with 0.5 McFarland solution that was prepared prior to the test. The bacterial culture was swabbed onto Mueller-Hinton Agar (MHA) using sterile cotton swab. Commercial antibiotic disks were then embedded onto the inoculated agar by using Oxoid™ Antimicrobial Susceptibility Disk Dispenser. Then, the inoculated agar were incubated at 37^{o}C for 18 to 24 hours.

The antibiotic tested were doxycycline (30 µg), chloramphenicol (30 µg), ceftriazone (30 µg), norfloxazin (10 µg), cephalotin (10 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), kanamycin (30 µg), imipenem (10 µg), erythromycin (15 µg), tobramycin (10 µg), streptomycin (10 µg), sulphamethoxazole (30 µg), ampicillin (10 U) and penicillin (10 U). After the incubation, the diameters of complete zone of inhibition were measured using standardized rule and were analyzed and grouped as susceptible, intermediate and resistant using standard interpretative table by Clinical Laboratory Standards Institute. Once groups, the Multiple Antibiotic Resistant (MAR) Index were calculated using the following formula:

$$MAR\ Index = \frac{number\ of\ antibiotic\ to\ which\ an\ isolate\ is\ resistant}{total\ number\ of\ antibiotics\ tested}$$

2.7 Genotyping of *B. cereus* associated with sago processing in Sarawak

2.7.1 Enterobacteria Repetitive Intergenic Consensus Sequences (ERIC) PCR

ERIC-PCR of genomic *B. cereus* was conducted according to Merzougui *et al.* (2013). The primer pairs used were 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and 5′-AAGTAAGTGACTGGGGTGAGCG-3′. To prepare 25 µL of PCR mixture, 1.0 M of forward primer, 1.0 M of reverse primer, 3.0 l of DNA template, 5.0 µL of 5×Taq PCR buffer, 0.2 mM dNTP, 2.0 mM $MgCl_2$, and 1.0 unit of Taq DNA polymerase (Promega) were mixed together. The PCR reaction was carried out according to the condition in the Table 2.4. The PCR products were then separated on 2% (w/v) agarose gel with 100 bp DNA ladder (Vivantis) for 90 min. After that, the gel was stained with ethidium bromide and viewed under a UV transilluminator (Maestrogen). The DNA band patterns were analyzed and a dendrogram was generated for the *B. cereus* isolates by using BioNumerics 7.6.2 software program (Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair group method (UPGMA). Simpson's index of diversity, D for ERIC-PCR was also calculated.

Table 2.4: Condition of ERIC PCR of *B. cereus*.

PCR steps	Temperature (°C)	Duration (minute)	Cycles
Initial denaturation	95	5.00	1
Denaturation	90	0.50	30
Annealing	50	0.50	30
Elongation	52	1.00	30
Final Extension	72	8.00	1

2.7.2 Pulsed-field gel electrophoresis (PFGE)

2.7.2.1 Preparation of agarose plug

In the present study, PFGE protocol was adapted and optimized from Merzougui *et al.* (2013). Twenty *B. cereus* pure isolates were randomly chosen and inoculated in Luria Bertani (LB) broth for 24 hours at 37°C. One milliliter of Tris-EDTA (TE) buffer (pH8, 0.01M) was dispensed into sterile 15 mL screw-capped Falcon tube. Then, 1 mL of overnight culture was suspended into 1 mL of TE buffer and adjusted into standardized suspension of the optical

density of 5.0 – 5.5 using a spectrometer machine. Two hundred microliter of adjusted cell suspension were transferred into 1.5 mL microcentrifuge tube. 10 µL of lysozyme (20 mg/mL) were added to the cell suspension and was incubated for 15 minutes at 37˚C. After incubation, 10 µL of proteinase K (1 mg/mL) were added, mixed gently and immediately 200 µL of melted 1% Seakem Gold Agarose were added to the suspension. After that, approximately 100 µL of the suspension were added to the plug mold and were allowed to solidify for 10 to 15 minutes.

2.7.2.2 Lysis of cells in agarose plugs

One point five milliliter of cell lysis buffer were dispensed into 1.5 mL microcentrifuge tube and labelled accordingly. The solidified plugs were pushed out into appropriate labelled tube and incubated into 54 ^{o}C water bath with constant 150 rpm agitation two hours.

2.7.2.3 Washing of agarose plugs

Upon incubation, the cell lysis buffer was removed. The plugs were washed twice with 10 mL of double sterile distilled water and four time with TE buffer for 15 minutes for each wash. Every wash was performed with 150 rpm agitation at room temperature.

2.7.2.4 Restriction of agarose plugs

B. cereus agarose plugs including *Salmonella* Braenderup H9812 universal standard plug were removed from TE buffer and cut into 1.5 mm slice using razor blade. All the plugs were transferred into new 1.5 mL microtube. Then, restriction mastermix for *B. cereus* agarose plugs including *Salmonella* Braenderup H9812 universal standard plug were prepared and the mixture for each microtube was shown in Table 2.5.

Table 2.5: Restriction mixture for each microtube

Reagent	*B. cereus* isolates	*Salmonella* Braenderup H9812 universal standard
Molecular grade water	177 µL	175 µL
10X restriction buffer	20 µL	20 µL
Restriction endonuclease *NotI* (*B. cereus* isolates) *XbaI* (H9812 standard)	3 U	5 U
Bovine serum albumin (BSA)	1 µL	1 µL

After that, 200 µL of restriction endonucleases were added to samples and standard tubes. All samples and standard tubes were incubated for 2 hours at 37 °C.

2.7.2.5 Gel electrophoresis

Samples plugs were subjected to gel electrophoresis. Electrophoresis was performed using CHEF-DRIII system in 2 liters of 0.5X TBE buffer. The chiller has defaulted to a 14°C set point, and buffer were allowed to circulate and cool completely prior to gel electrophoresis run according to the running parameters as shown in Table 2.6. After 20 hours of run time, the agarose gel was pre-stained with ethidium bromide and visualized with the aid of UV transilluminator (Maestrogen).

Table 2.6: Gel electrophoresis running parameters

Parameters	Unit
Voltage density	6 V / minute
Included angle	120 ^{o}
Initial switch time	2.4 s
Final switch time	60.8 s
Run time	20 hours
Ramping	Linear

CHAPTER 3

RESULTS

3.1 Prevalence of *B. cereus* associated with sago processing in Sarawak

3.1.1 Identification of *B. cereus* by polymerase chain reaction (PCR) assay

A total of 42 presumptive *B. cereus* were further confirmed by using PCR assay. *B. cereus* was detected by using PCR by targeting haemolysin gene (*hly*) of *B. cereus* with the product size of 185 bp. The gel picture of the PCR amplication was shown in Figure 3.1. Based on the PCR assay detection, it was revealed that *B. cereus* were present in 35% (42/120) of the samples consist of bark swab, sago pith, starch slurry, sago milk, sago flour and sago effluent collected from the both farms. Of these positive samples, 20% (12/60) and 50% (30/60) of *B. cereus* were present in sago processing mill A and sago processing mill B respectively. The prevalence of *B. cereus* from both sago processing mill A and sago processing B were shown in Table 3.

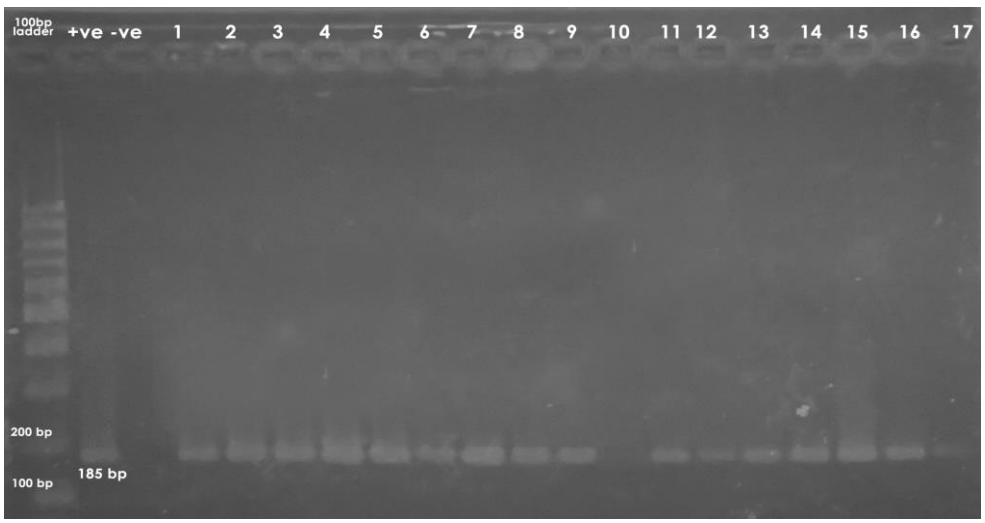

Figure 3.1: The amplicon obtained from specific PCR targeting haemolysin gene of *B. cereus* at 185 bp on the 2% agarose gel. Lane 100 bp ladder = 100 bp DNA ladder. +ve : Positive control. –ve : Negative control. Lane 1 – 10: Sago water effluent sample. Lane 11 – 17: Sago sample from debarking stage.

Table 3.1: The prevalence of *B. cereus* from sago processing A and sago processing B based on PCR assay.

Type of sample	**Processing steps**	**Sago processing mill A**		**Sago processing mill B**		**TOTAL**
		Prevalence (%)	Range of concentration (CFU/mL)	Prevalence (%)	Range of concentration (CFU/mL)	
Bark swab	Debarking	6 % (6/10)	5.3×10^2-3.4×10^4	90% (9/10)	1.2×10^3-5.3×10^5	75% (15/20)
Sago pith	Pulping	30% (3/10)	4.4×10^2-5.3×10^4	60% (6/10)	6.5×10^3-2.3×10^4	45% (9/20)
Starch slurry	Starch extraction	0% (0/10)	-	40% (4/10)	3.2×10^2-2.7×10^4	20% (4/20)
Sago milk	Drying	0% (0/10)	-	20% (2/10)	2.4×10^2-2.5×10^3	10% (2/20)
Sago flour	Packaging	0% (0/10)	-	0% (0/10)	-	
Sago effluent	Waste discharge	30% (3/10)	4.2×10^3-3.3×10^5	90% (9/10)	3.6×10^4-6.5×10^5	60% (12/20)
TOTAL		20% (12/60)		50% (30/60)		35% (42/60)

- : Not available

The prevalence of *B. cereus* differ based on the sampling location as well as types of samples. Sago processing mill B has higher prevalence, 50% (30/60) when compared to sago processing mill B, 20% (12/60). *B. cereus* were detected in debarking (60%), pulping (30%) and waste discharging point (30%) stage for sago processing mill A where as in sago processing mill B, *B. cereus* were detected in debarking (90%), pulping (60%), starch extraction (40%), drying (20%) and waste discharging point (90%). However, there are no *B. cereus* were detected in drying and packaging stage of sago processing mill A and in packaging stage of sago processing mill B.

Debarking stage at sago processing mill B has the highest prevalence, 90% (9/10), followed by starch extraction, discharge and pulping in sago processing B which the prevalence are 90% (9/10), 90% (9/10) and 60% (6/10) respectively. Drying and packaging in sago processing mill A and packaging in sago processing mill B indicate the lowest prevalence where there are no detection of *B. cereus.*

The enumeration of *B. cereus* were carried out by using standard plate count method. Peacock blue colonies with precipitate and peacock blue medium were enumerated and calculated. The standard plate count (in CFU/mL) of *B. cereus* from sago processing mill A and sago processing mill were also shown in Table 3.1. The *B. cereus* in bark swab in debarking stage, sago pith in pulping stage and sago effluent from waste discharging point of sago mill A ranged between 5.3×10^2 to 3.4×10^4 CFU/mL, 4.4×10^2 CFU/mL to 5.3×10^4 and 4.2×10^3 to 3.3×10^5 CFU/mL respectively whereas *B. cereus* in debarking, pulping, starch extraction, drying and waste discharging stage have plate count ranged between 1.2×10^3 - 5.3×10^5, 6.5×10^3 - 2.3×10^4, 3.2×10^2 - 2.7×10^4, 2.4×10^2 - 2.5×10^3 and 3.6×10^4 - 6.5×10^5 CFU/mL respectively. However, *B. cereus* were not detected in drying and packaging stage in sago processing mill A and packaging stage in sago processing mill B.

From all the samples collected, waste discharging point at sago processing mill B has the highest concentration of *B. cereus*, followed by waste discharging point at sago processing A.

3.2 Antibiotic Susceptibility Test

Forty-two *B. cereus* isolates were subjected to antibiotic susceptibility test. The result for the antibiotic susceptibility test (AST) was tabulated in Table 3.2, 3.3 and 3.4. The percentage of antibiotic resistancy-susceptibility of *B. cereus* was also displayed in form of graph in Figure 3.2.

Table 3.2: Percentage of bacterial resistance of all *B. cereus* isolates based on antibiotic

Antibiotics	**Total of isolates tested**	**Number of resistant isolates**	**Percentage of Resistance (%)**
Doxycycline (DO)	42	19	45.2
Chloramphenicol (C)	42	22	52.4
Ceftriazone (CRO)	42	26	61.9
Norfloxazin (NOR)	42	0	0.0
Cephalotin (KF)	42	37	88.1
Nitrofurantoin (F)	42	15	35.7
Kanamycin (K)	42	12	28.6
Imipenem (IMP)	42	0	0.0
Erythromycin (E)	42	14	33.3
Tobramycin (TOB)	42	2	4.8
Streptomycin (S)	42	12	28.6
Sulphamethoxazole / Trimtoprim (SXT)	42	8	19.0
Ampicillin (AMP)	42	42	100.0
Penicillin (P)	42	42	100.0

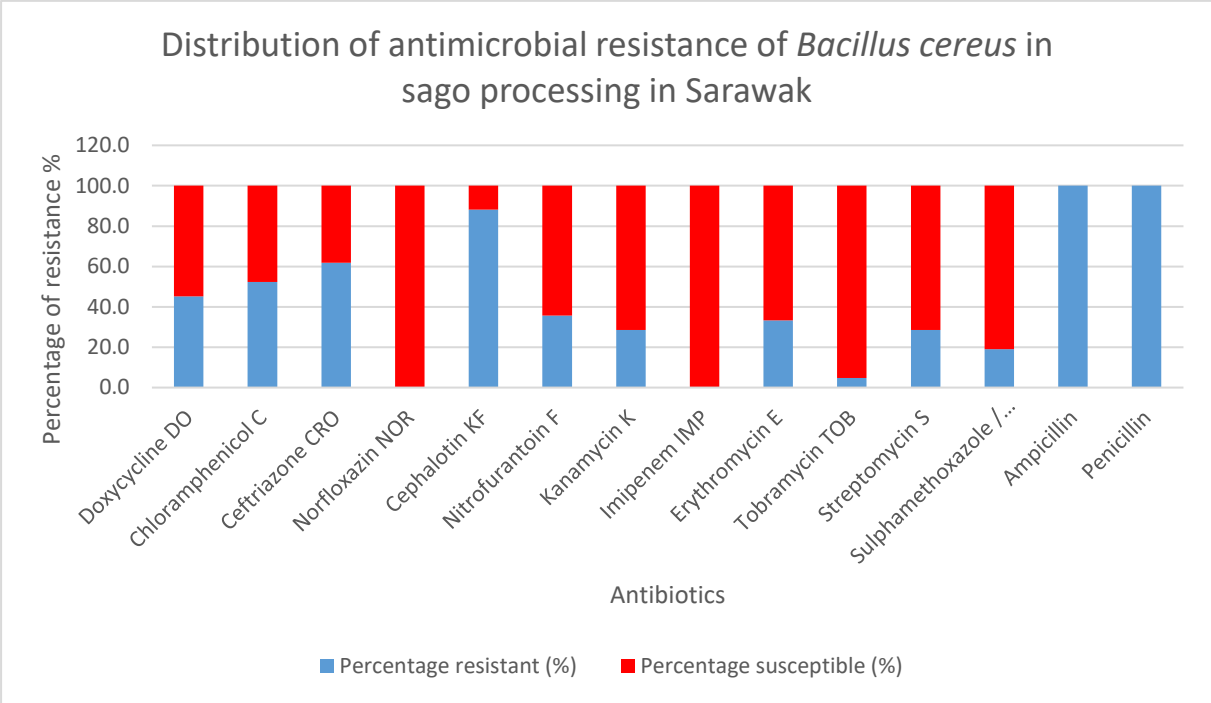

Figure 3.2: Percentage of antibiotic resistancy-susceptibility of *B. cereus* in sago processing in Sarawak.

Based on Table 3.2 and Figure 3.2, the highest resistance recorded were 100% (42/42) which were against penicillin and ampicillin, followed by cephalotin 88.1% (37/42), ceftriazone 61.9% (26/42), chloramphenicol 52.4% (22/42), doxycycline 45.2% (19/42), nitrofurantoin 35.7% (15/42), kanamycin 28.6% (12/42), streptomycin 28.6% (12/42), sulphamethoxazole 19.0% (8/42), tobramycin 4.8% (2/42), imipenem 0.0% (0/42) and norfloxazin 0.0% (0/42).

Table 3.3: Percentage of antibiotic resistance of *B. cereus* isolated at sago processing mill A

Antibiotics	**Percentage of bacterial resistance (%)**						
	Debark-ing n=6	Pulp-ing n=3	Starch extract-ion n=0	Dry-ing n=0	Packa-ging n=0	Waste dischar-ging point n=3	TOTAL
Doxycycline (DO)	66.7 (4/6)	100.0 (3/3)	-	-	-	33.3 (1/3)	66.7 (8/12)
Chloramphenicol (C)	66.7 (4/6)	66.6 (2/3)	-	-	-	33.3 (1/3)	58.3 (7/12)
Ceftriazone (CRO)	83.3 (5/6)	66.6 (2/3)	-	-	-	66.6 (2/3)	75.0 (9/12)
Norfloxazin (NOR)	0.0 (0/6)	0.0 (0/3)	-	-	-	0.0 (0/3)	0.0 (0/12)
Cephalotin (KF)	83.3 (5/6)	100.0 (3/3)	-	-	-	100.0 (3/3)	91.7 (11/12)
Nitrofurantoin (F)	66.7 (4/6)	33.3 (1/3)	-	-	-	0.0 (0/3)	41.7 (5/12)
Kanamycin (K)	16.7 (1/6)	100.0 (3/3)	-	-	-	0.0 (0/3)	33.3 (4/12)
Imipenem (IMP)	0.0 (0/6)	0.0 (0/3)	-	-	-	0.0 (0/3)	0.0 (0/12)
Erythromycin (E)	33.3 (2/6)	100.0 (3/3)	-	-	-	0.0 (0/3)	41.7 (5/12)
Tobramycin (TOB)	16.7 (1/6)	0.0 (0/3)	-	-	-	33.3 (1/3)	16.7 (2/12)
Streptomycin (S)	0.0 (0/6)	100.0 (3/3)	-	-	-	0.0 (0/3)	25.0 (3/12)
Sulphamethoxa-zole / Trimtoprim (SXT)	33.3 (1/6)	33.3 (1/3)	-	-	-	0.0 (0/3)	16.7 (2/12)
Ampicillin (AMP)	100.0 (6/6)	100.0 (3/3)	-	-	-	100.0 (3/3)	100.0 (12/12)
Penicillin (P)	100.0 (6/6)	100.0 (3/3)	-	-	-	100.0 (3/3)	100.0 (12/12)

Table 3.4: Percentage of antibiotic resistance of *B. cereus* isolated at sago processing mill B

Antibiotics	**Percentage of bacterial resistance**						
	Debar-king n=9	Pulping n=6	Starch extrac-tion n=4	Dry-ing n=2	Packa-ging n=0	Waste dischar-ging point n=9	TOTAL
Doxycycline (DO)	11.1 (1/9)	33.3 (2/6)	50.0 (2/4)	100.0 (2/2)	-	44.4 (4/9)	33.7 (11/30)
Chloramphenicol (C)	33.3 (3/9)	50.0 (3/6)	100.0 (4/4)	50.0 (1/2)	-	44.4 (4/9)	50.0 (15/30)
Ceftriazone (CRO)	55.6 (5/9)	66.7 (4/6)	25.0 (1/4)	0.0 (0/2)	-	77.8 (7/9)	56.7 (17/30)
Norfloxazin (NOR)	0.0 (0/9)	0.0 (0/6)	0.0 (0/4)	0.0 (0/2)	-	0.0 (0/9)	0.0 (0/30)
Cephalotin (KF)	88.9 (8/9)	100.0 (6/6)	0.0 (0/4)	0.0 (2/2)	-	77.8 (7/9)	76.7 (23/30)
Nitrofurantoin (F)	44.4 (4/9)	33.3 (2/6)	0.0 (0/4)	50.0 (1/2)	-	33.3 (3/9)	33.3 (10/30)
Kanamycin (K)	22.2 (2/9)	16.7 (1/6)	0.0 (0/4)	0.0 (0/2)	-	55.6 (5/9)	26.7 (8/30)
Imipenem (IMP)	0.0 (0/9)	0.0 (0/6)	0.0 (0/4)	0.0 (0/2)	-	0.0 (0/9)	0.0 (0/30)
Erythromycin (E)	44.4 (4/9)	16.7 (1/6)	0.0 (0/4)	0.0 (0/2)	-	44.4 (4/9)	30.0 (9/30)
Tobramycin (TOB)	0.0 (0/9)	0.0 (0/6)	0.0 (0/4)	0.0 (0/2)	-	0.0 (0/9)	0.0 (0/30)
Streptomycin (S)	33.3 (3/9)	16.7 (1/6)	25.0 (1/4)	0.0 (0/2)	-	44.4 (4/9)	30.0 (9/30)
Sulphamethoxa-zole / Trimtoprim (SXT)	11.1 (1/9)	16.7 (1/6)	50.0 (2/4)	0.0 (0/2)	-	22.2 (2/9)	20.0 (6/30)
Ampicillin (AMP)	100.0 (9/9)	100.0 (6/6)	100.0 (4/4)	100.0 (2/2)	-	100.0 (9/9)	100.0 (30/30)
Penicillin (P)	100.0 (9/9)	100.0 (6/6)	100.0 (4/4)	100.0 (2/2)	-	100.0 (9/9)	100.0 (30/30)

B. cereus from sago processing mill A were 100% (12/12) resistant to penicillin and ampicillin and 100% (12/12) susceptible to norfloxazin and imipenem. In this farm, *B. cereus* are 91.7% (11/12), 75.0% (9/12), 66.7% (8/12), 58.3% (7/12), 41.7% (5/12), 41.7% (5/12), 33.3% (4/12), 25.0% (3/12), 16.7% (2/12) and 16.7% (2/12) were resistant to cephalotin, ceftriazone, doxycycline, chloramphenicol, nitrofurantoin, kanamycin, streptomycin, sulphamethoxazole, tobramycin respectively. The percentage of antibiotic resistance of *B. cereus* isolated at sago processing mill A were summarized in Table 3.3.

For sago processing mill B, the highest resistance recorded were 100% (30/30) which were against penicillin and ampicillin, followed by cephalotin 76.7% (23/30), ceftriazone 56.7% (17/30), chloramphenicol 50.0% (15/30), doxycycline 33.7% (11/30), nitrofurantoin 33.3% (10/30), kanamycin 26.7% (8/30), streptomycin 30.0% (9/30), sulphamethoxazole 20.0% (6/30), tobramycin 0.0% (0/30), imipenem 0.0% (0/30) and norfloxazin 0.0% (0/30). The percentage of antibiotic resistance of *B. cereus* isolated at sago processing mill B were summarized in Table 3.4.

Figure 3.3 and 3.4 show the percentage of antibiotic resistance of *B. cereus* in every stage of processing in sago processing mill A and B respectively. *B. cereus* was isolated from debarking, pulping and waste discharging stage in sago processing mill A were as in sago processing mill B, *B. cereus* was isolated from debarking, pulping, starch extraction, drying and waste discharging stage. For debarking stage in sago processing mill A, *B. cereus* was 100% (6/6) resistant to penicillin and ampicillin, followed by cephalotin and ceftriazone which both were 83.3% (5/6) resistant, doxycycline, nitrofurantoin and chloramphenicol, 66.7% (4/6), erythromycin and sulphamethoxazole, 33.3% (2/6), kanamycin and tobramycin 16.7% (1/6).

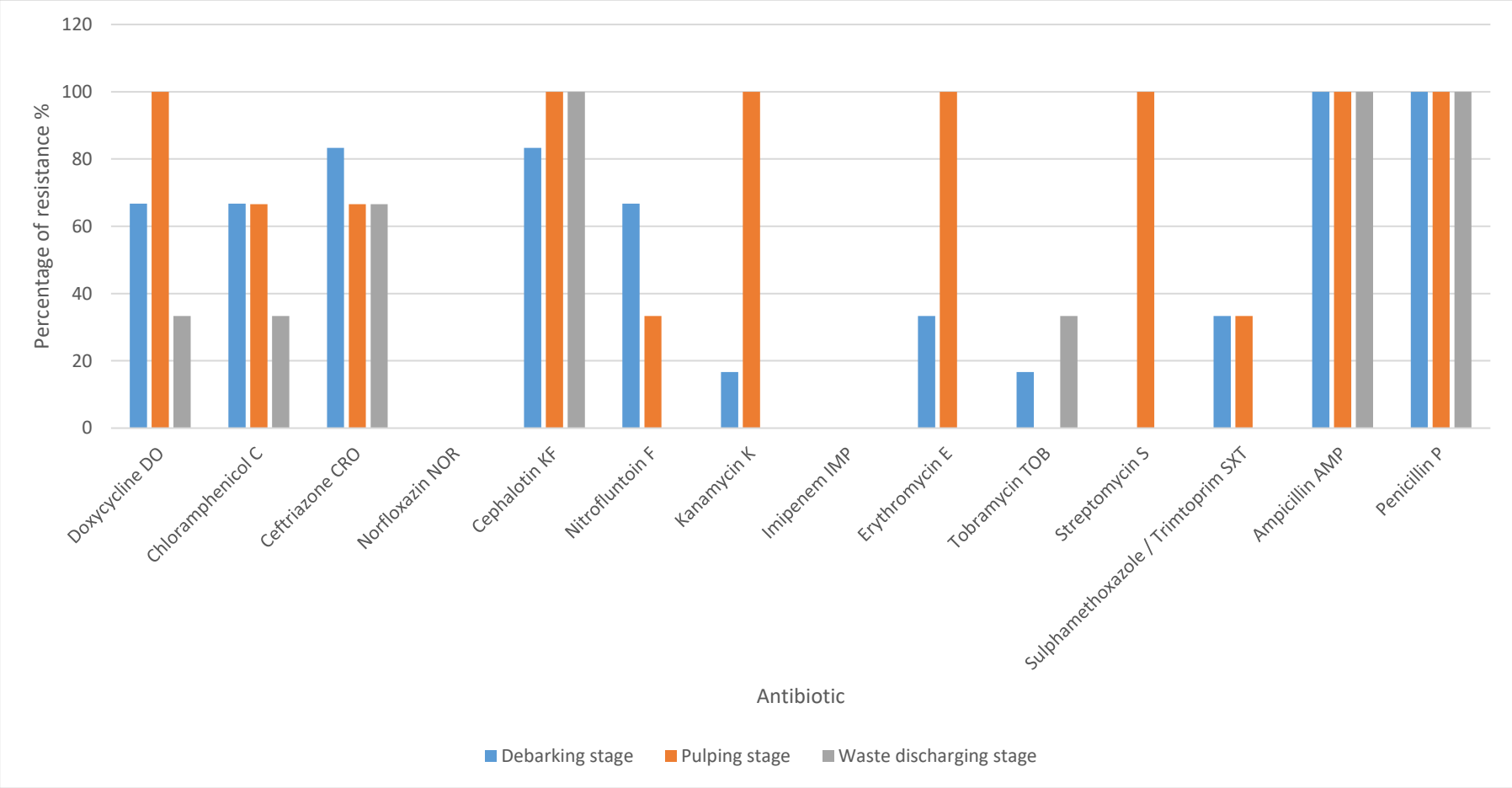

Figure 3.3: The percentage of antibiotic resistance of *B. cereus* in every stage of processing in sago processing mill A.

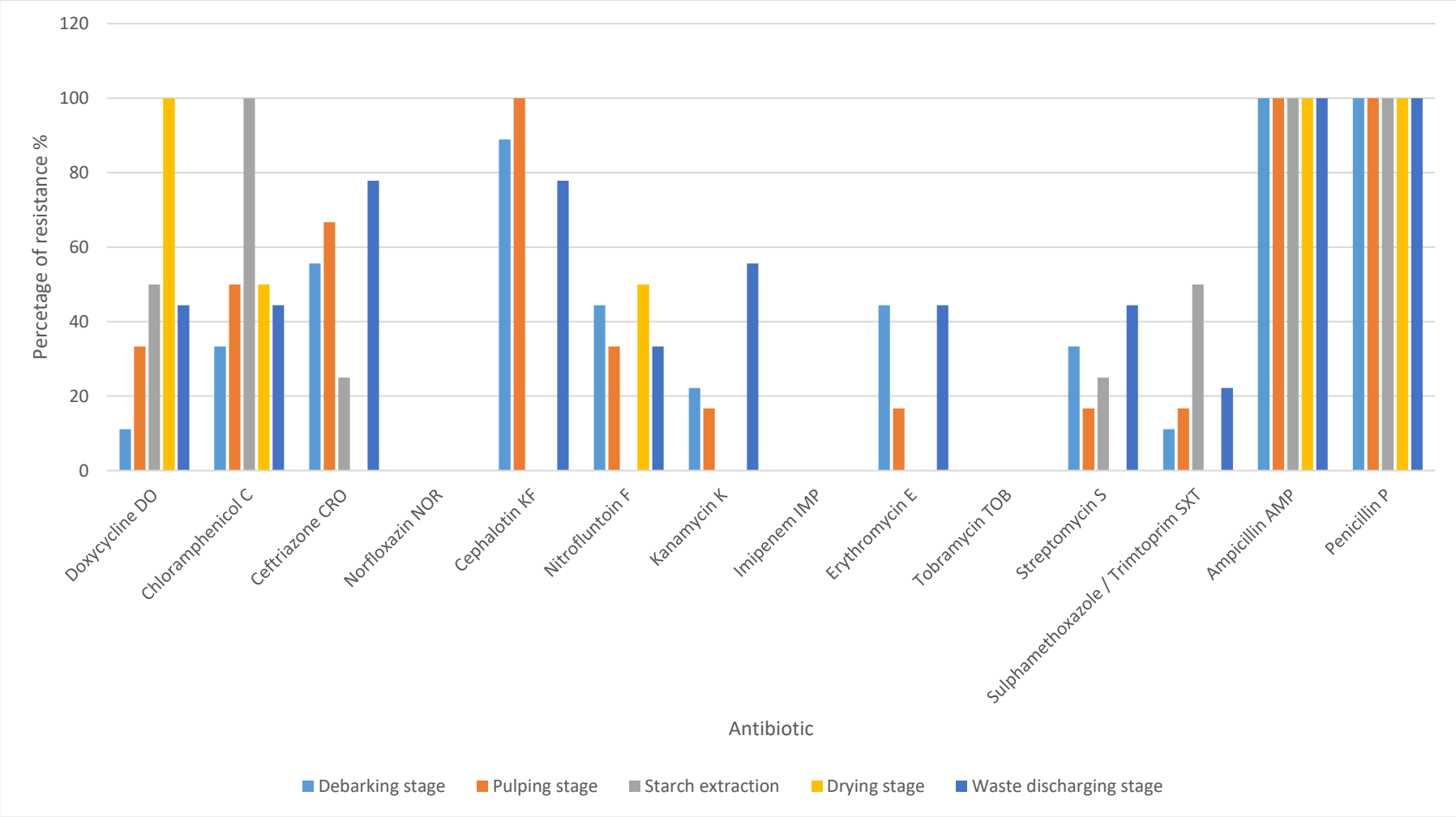

Figure 3.4: The percentage of antibiotic resistance of *B. cereus* in every stage of processing in sago processing mill B

However, *B. cereus* was 100% susceptible to streptomycin, imipenem and norfloxazin. For pulping stage, *B. cereus* was 100% (3/3) resistant to doxycycline, cephalotin, kanamycin, erythromycin, streptomycin, penicillin and ampicillin, 66.6% (2/3) resistant to chloramphenicol and ceftriazone and 33.3% (1/3) resistant to nitrofurantoin and sulphamethoxazole/trimtoprim. On the other hand, *B. cereus* isolated from this stage was 100% susceptible to imipenem, tobramycin and norfloxazine. Other than that, *B. cereus* isolated waste discharging stage was 100% (3/3) resistant to cephalotin, ampicillin and penicillin, 66.6% (2/3) resistant to ceftriazone, 33.3% (1/3) resistant to doxycycline, chloramphenicol and tobramycin. On the other hand, *B. cereus* isolated from this stage were 100% susceptible to norfloxazin, nitrofurantoin, kanamycin, imipenem, erythromycin, streptomycin and sulphamethoxazole / trimtoprim.

For sago processing mill B, *B. cereus* isolated from debarking stage was most resistant to ampicillin and penicillin, 100% (9/9), followed by cephalotin, 88.9% (8/9), ceftriozone, 55.6% (5/9), nitrofurantoin and erythromycin, 44.4% (4/9), chloramphenicol and streptomycin, 33.3% (3/9), kanamycin, 22.2% (2/9) and doxycycline and sulphametoxazole/trimtoprim, 11.1% (1/9). However, *B. cereus* was 100% susceptible to norfloxazin, imipenem and tobramycin.

In this study, the antibiotic resistant patterns of all isolates were also determined to monitor the spread of antibiotic resistance. Thirty-two antibiotic resistance pattern were observed through this study as stated in Table 4.5. The antibiotic resistance were highly variable; 7.1% (3/42) of the isolates have resistance towards one antibiotic and 92.9% (39/42) isolates have multiple antibiotic resistance. The MAR index for all isolates were also varies, ranged from 0.083 to 0.750. MAR index higher than 0.20 indicates that the particular organism is originated from high-risk sources of contamination, where antibiotics are often used. MAR

index less than or equal to 0.20, indicates that the isolate is originated from environment where antibiotics are seldom or never used (Krumperman, 1983; Pandove *et al.*, 2013). Overall, 23.8% (10/42) isolates have MAR index less than 0.2 where as 76.2% (32/42) isolates have MAR index more than 0.2. MAR index of *B. cereus* in this study was summarized in Figure 3.5.

For sago processing mill A, 8.3% (1/12) have *B. cereus* isolate resistant to one antibiotic where as 91.7% (11/12) isolates have resistant towards multiple antibiotics. MAR index of *B. cereus* isolate in sago processing mill A ranged between 0.083 and 0.750. 8.3% (1/12) isolates has MAR index less than 0.2, whereas 91.7% (11/12) isolates have MAR index more than 0.2. On the other hand, for sago processing mill B, MAR index less than 0.2 and more than 0.2 were 30% (9/30) isolates and 70% (21/30) isolates respectively.

Table 3.5: Antibiogram of *B. cereus* in sago processing in Sarawak

Location	Isolate	Processing step	Sample	Antibiotic Resistant Pattern	Pattern	MAR Index
SAGO PROCESSING MILL A	SPA 11	Debarking	Bark swab	DO/C/F/KF	1	0.333
	SPA 12			DO/C/CRO/KF	2	0.333
	SPA 14			DO/CRO/F/KF	3	0.333
	SPA 16			CRO/K/E/SXT	4	0.333
	SPA 17			C/CRO/F/KF/E	5	0.417
	SPA 18			DO/C/CRO/F/KF	6	0.417
	SPA 22	Pulping	Sago pith	DO/C/CRO/F/KF/K/E/S/SXT	7	0.750
	SPA 28			DO/CRO/KF/K/E/S	8	0.500
	SPA 29			DO/C/KF/K/E/S	9	0.500
	SPA 64	Waste discharging	Sago effluent	DO/C/CRO/KF	10	0.333
	SPA 65			KF	11	0.083
	SPA 69			CRO/KF/TOB	12	0.250
SAGO PROCESSING MILL B	SPB 11	Debarking	Bark swab	DO/C/CRO/F/KF	4	0.500
	SPB 12			KF	11	0.083
	SPB 13			KF/SXT	13	0.167
	SPB 14			CRO/KF/E	14	0.250
	SPB 15			KF/K/S	15	0.250
	SPB 16			F/KF/K/S	16	0.333
	SPB 17			C/CRO/F/KF/E	6	0.417
	SPB 18			C/CRO/F/KF	17	0.333
	SPB 19			CRO/E	18	0.167
	SPB 21	Pulping	Sago pith	DO/C/F/KF/K/E/S/SXT	19	0.667
	SPB 22			CRO/F	20	0.167
	SPB 24			CRO/KF	21	0.167
	SPB 27			DO/C/CRO/F/KF	4	0.417

SAGO PROCESSING MILL B	SPB 28			C/CRO/KF	22	0.250
	SPB 29			KF/TOB	23	0.167
	SPB 31	Starch extraction	Starch slurry	C/CRO/SXT	24	0.250
	SPB 35			C/KF/SXT	25	0.250
	SPB 37			DO/C/KF/SXT	26	0.333
	SPB 39			DO/C/KF	27	0.250
	SPB 47	Drying	Sago milk	DO/KF	28	0.167
	SPB 48			DO/C/F/KF	1	0.333
	SPB 61	Waste discharging	Sago effluent	CRO/K/E/S/SXT	29	0.417
	SPB 62			CRO/F	20	0.167
	SPB 63			DO/C/CRO/F/KF	4	0.417
	SPB 64			DO/C/CRO/F/KF/K/E/S/SXT	7	0.750
	SPB 65			DO/CRO/KF/K/E/S	30	0.500
	SPB 66			C/KF/K/E/S	31	0.417
	SPB 67			DO/C/CRO/KF	10	0.333
	SPB 68			KF	11	0.083
	SPB 69			CRO/KF/K	32	0.250

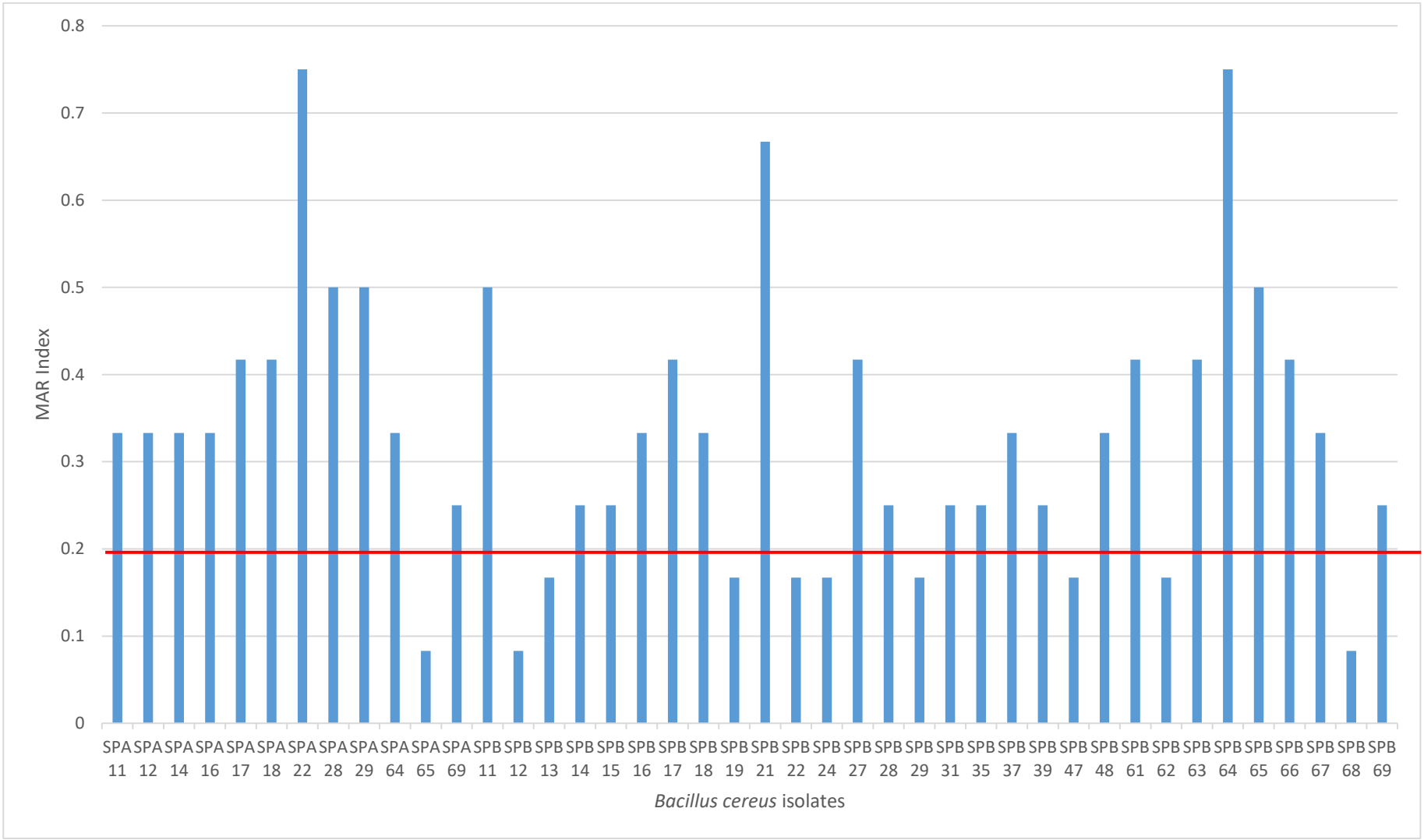

Figure 3.5: MAR index of *B. cereus* in sago processing in Sarawak

3.3 Genotyping of *B. cereus* associated with sago processing in Sarawak by ERIC-PCR and PFGE

3.3.1 ERIC-PCR

Fourty-two *B. cereus* isolates from sago processing A and B were subjected to ERIC-PCR. The objective of ERIC-PCR analysis in this study was to group these bacterial isolates based on its genetic diversities according to sampling sites. Based on the gel photograph in Figure 3.6, 3.7 and 3.8, the electrophoretic profile of DNA fragments obtained after ERIC-PCR amplification for both sago processing mills yielded 1 - 7 bands with size approximately 100 bp to 990 bp. A total of 35 DNA fingerprint profiles were produced in this analysis whereby 12 DNA fingerprint profiles from sago processing A and 23 DNA fingerprint profiles from sago processing B. The dendrogram shown in Figure 3.9 for sago processing mill A and Figure 3.10 for sago processing mill B were constructed by using Bionumerics 7.6.2 software.

ERIC-PCR banding profiles of sago processing mill A displayed high genetic diversity. Twelve different DNA fingerprint profiles and none of the isolates from sago processing A has indistinguishable DNA fingerprint profiles. These banding were presented in form of dendrogram as shown in Figure 3.9. Based on the dendrogram constructed *B. cereus* isolates from sago processing mill A were grouped into two major clusters; cluster A and cluster B. Cluster B is the largest cluster for sago processing mill A. It consists of 91.7% (11/12) isolates: 54.5% (6/11) isolates from debarking stage, 18.1% (2/11) isolates from pulping stage and 27.2% (3/11) isolates from waste discharging point. Cluster A on the other hand consists of 8.3% (1/12) isolate only which is from the pulping stage.

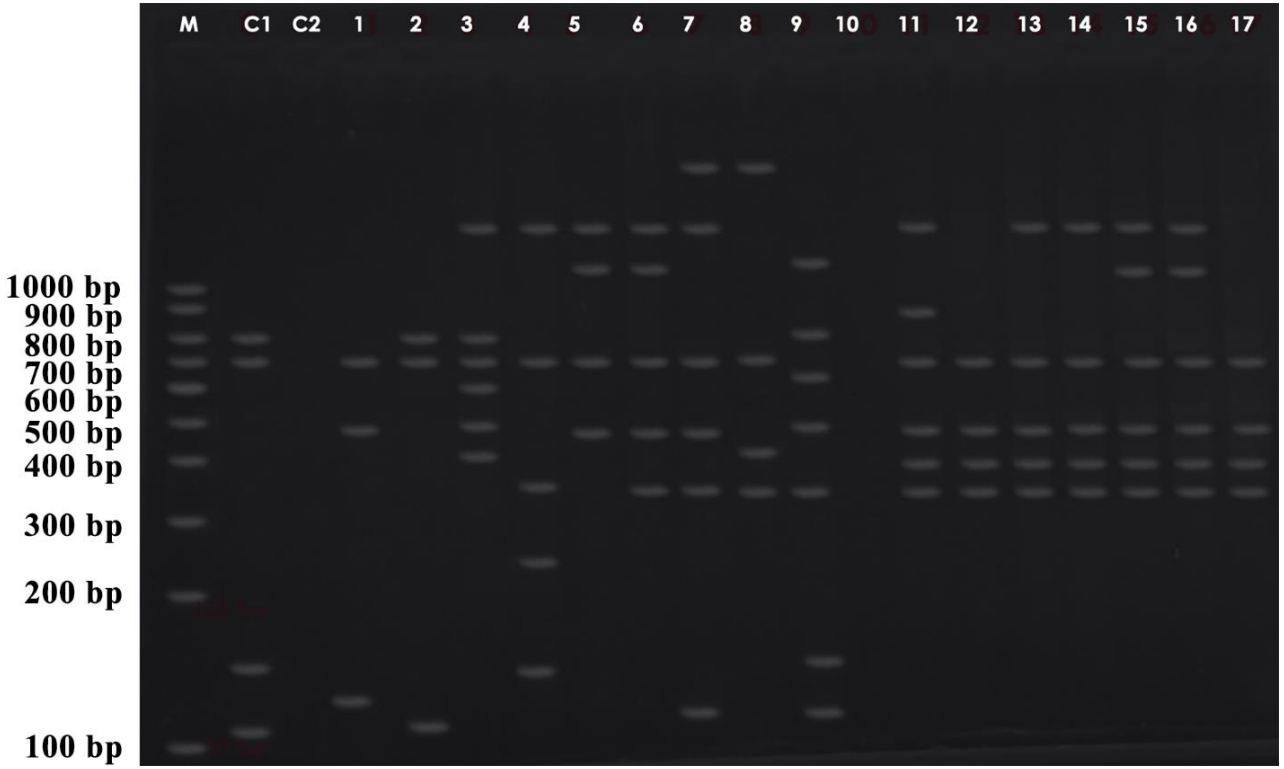

Figure 3.6: Banding profiles of ERIC PCR for sago processing mill A (lane 1-12) and B (lane 13-17). **Lane M:** 100 bp ladder (Vivantis, USA), **C1:** Positive control, **C2:** Negative control, **1:** SPA 11, **2:** SPA 12, **3:** SPA 14, **4:** SPA 16, **5:** SPA 17, **6:** SPA 18, **7:** SPA 22, **8:** SPA 28, **9:** SPA 29, **10:** SPA 64, **11:** SPA 65, **12:** SPA 69, **13:** SPB 11, **14:** SPB 12, **15:** SPB 13, **16:** SPB 14, **17:** SPB 15.

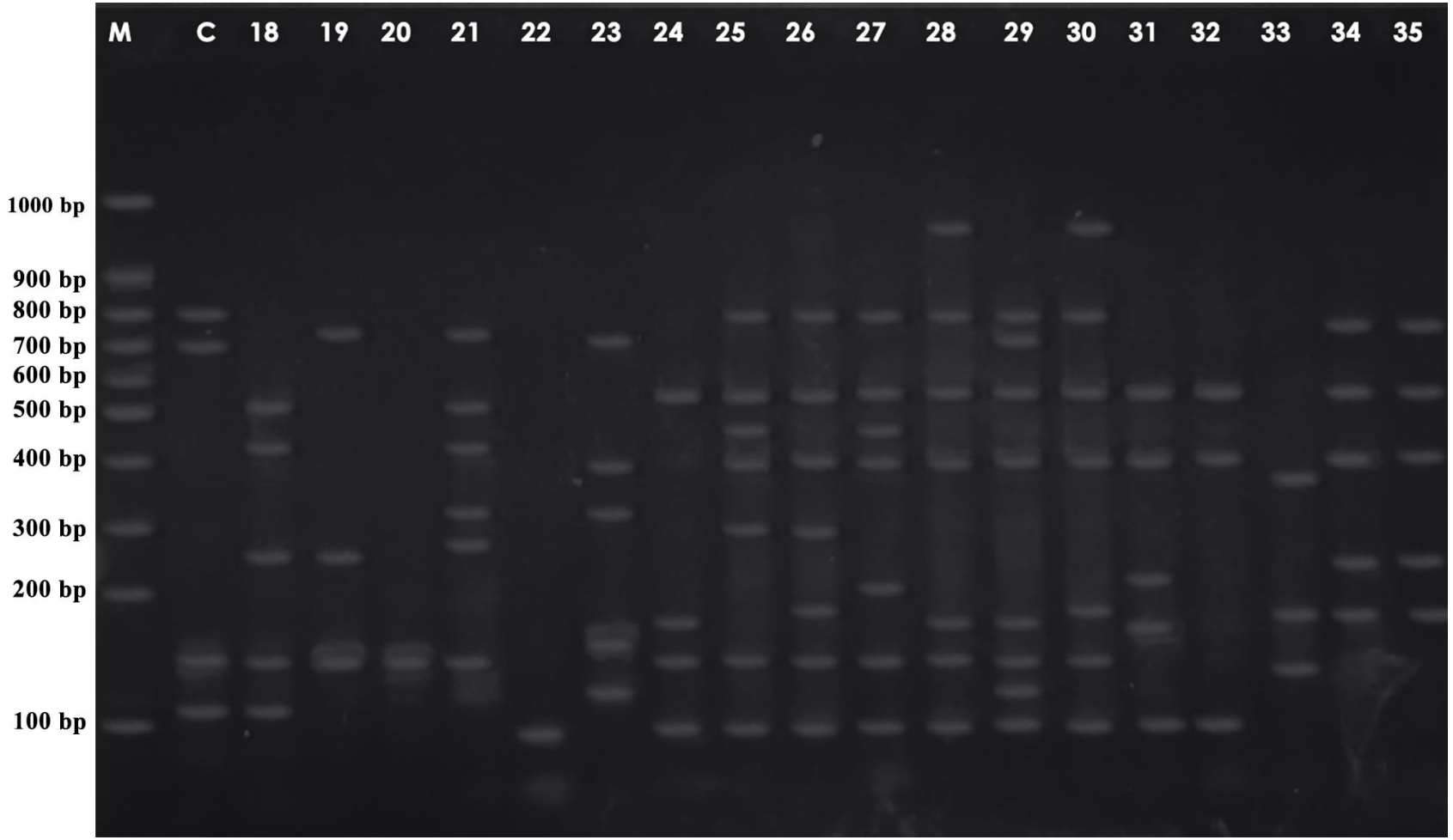

Figure 3.7: Banding profiles of ERIC PCR for sago processing mill B. **Lane M:** 100 bp ladder (Vivantis, USA), **C:** Positive control, **18:** SPB 16, **19:** SPB 17, **20:** SPB 18, **21:** SPB 19, **22:** SPB 21, **23:** SPB 22, **24:** SPB 24, **25:** SPB 27, **26:** SPB 28, **27:** SPB 29, **28:** SPB 31**, 29:** SPB 35**, 30:** SPB 37 **, 31:** SPB 39, **32:** SPB 47 **33:** SPB 48, **34:** SPB 61, **35:** SPB 62

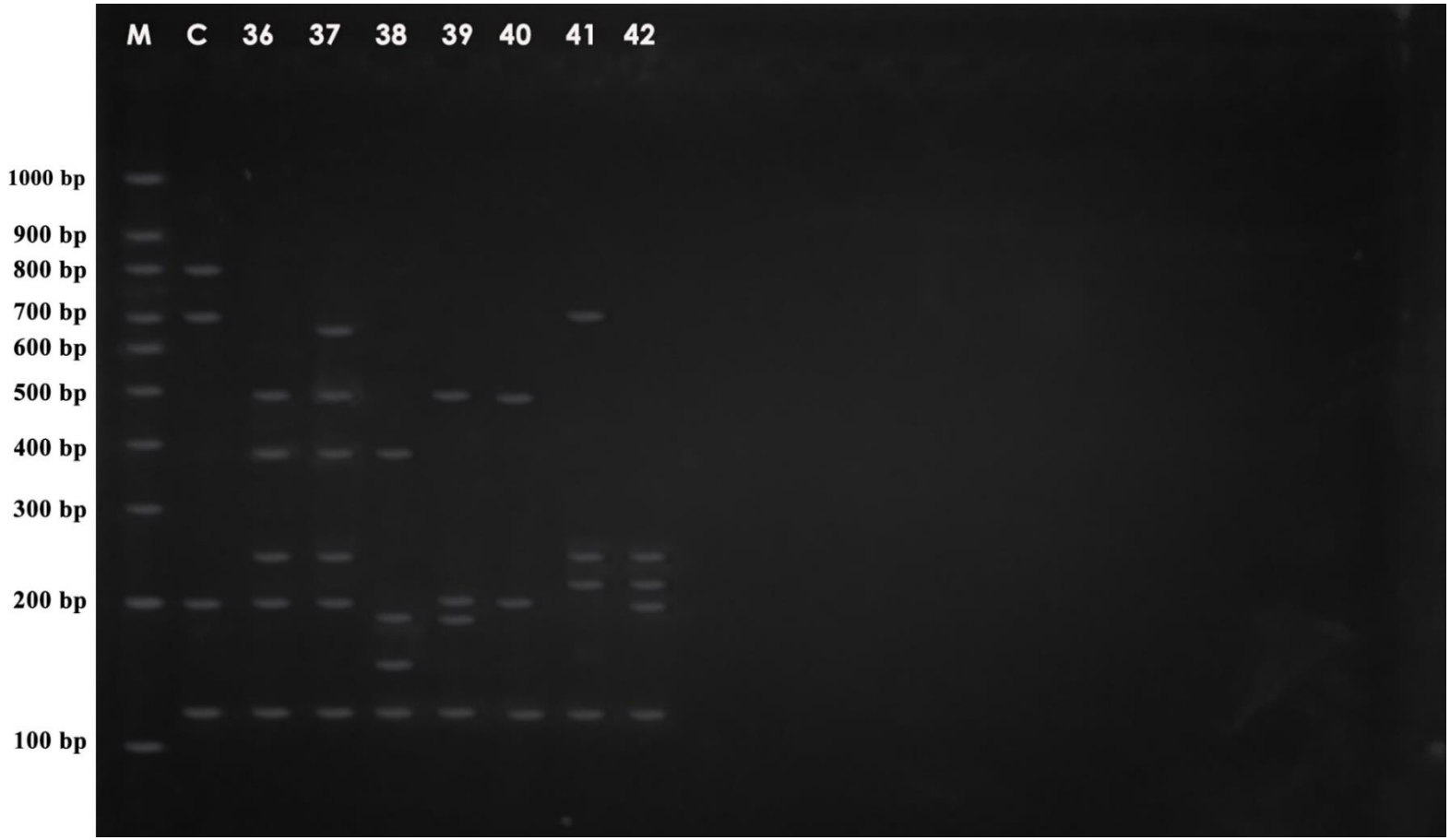

Figure 3.8: Banding profiles of ERIC PCR for sago processing mill B. **Lane M:** 100 bp ladder (Vivantis, USA), **C:** Positive control, **36:** SPB 63, **37:** SPB 64 **38:** SPB 65, **39:** SPB 66, **40:** SPB 67, **41:** SPB 68, **42:** SPB 6

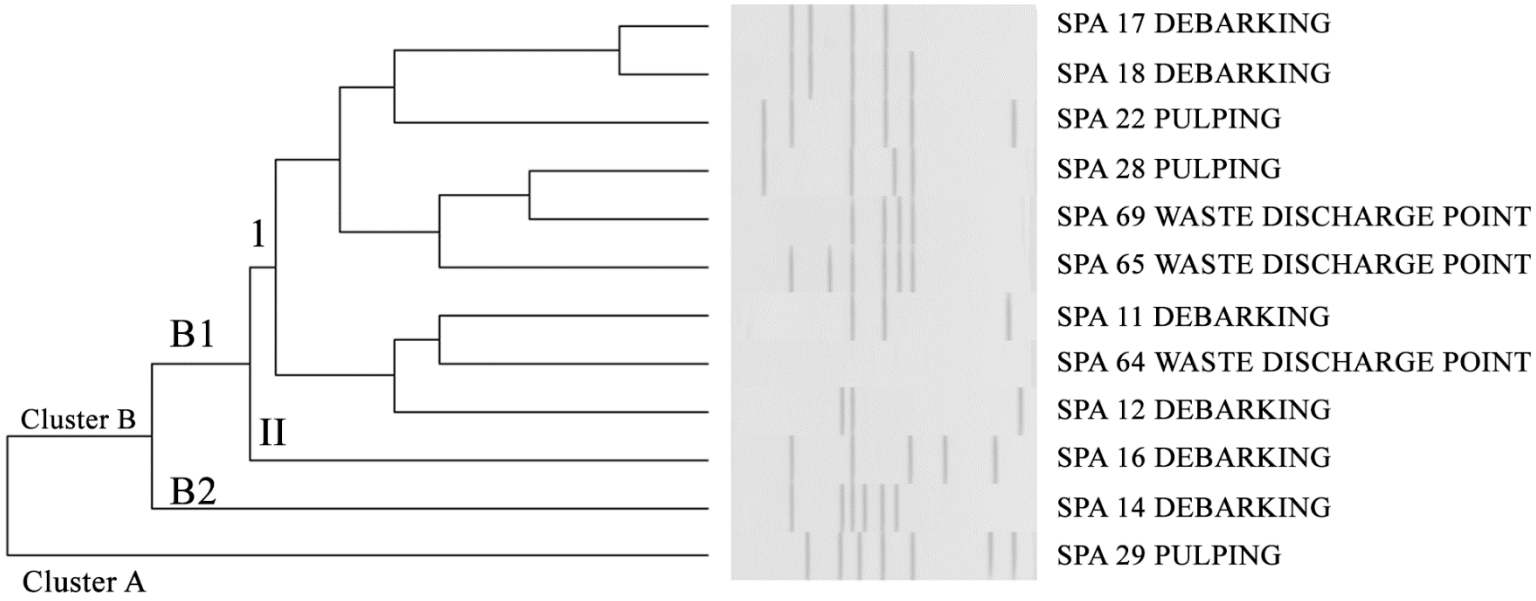

Figure 3.9: Dendrogram of ERIC-PCR profiles for sago processing mill A

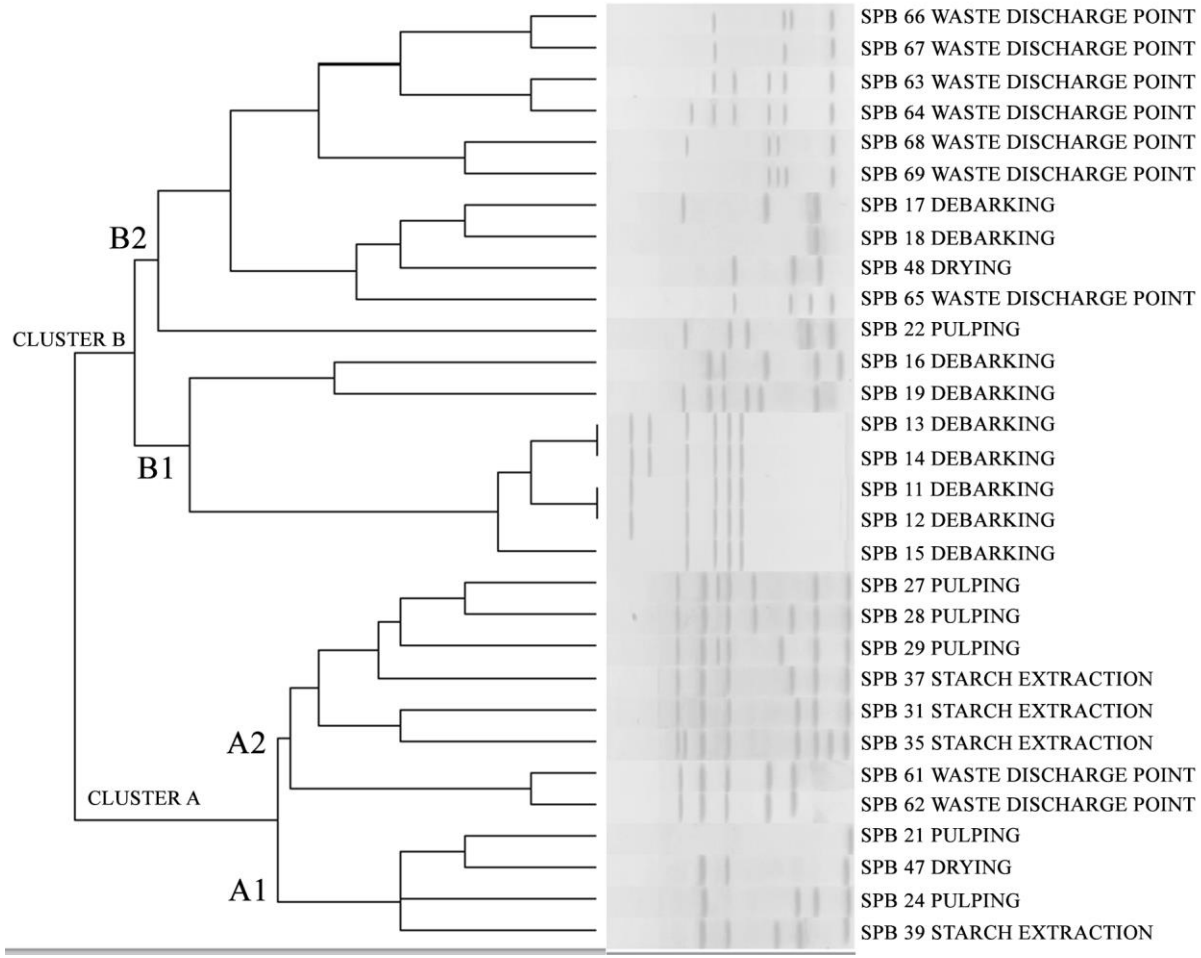

Figure 3.10: Dendrogram of ERIC-PCR profiles for sago processing mill B

ERIC-PCR banding profiles of sago processing mill B also displayed high genetic diversity. Twenty-six DNA fingerprint profiles were produced in this analysis and four pair (SPB 13 & SPB 14, SPB 11 & SPB 12, SPB 31 & SPB 37 and SPB 61 and SPB 62) of the isolates showed indistinguishable DNA fingerprint profiles. These banding were presented in form of dendrogram as shown in Figure 3.10. Based on the dendrogram constructed, *B. cereus* isolates from sago processing mill B were grouped into two major clusters; cluster A and cluster B. Cluster B is larger than cluster A for sago processing mill A. It consists of 60% (18/30) isolates: 50% (9/18) isolates from debarking stage, followed by waste discharging stage, 38.9% (7/18) and 5.6% (1/18) isolates for both drying and pulping stage. For cluster A, it consists of 40% (12/30) of the isolates where by 41.7% (5/12) isolates from pulping stage, 33.3% (4/12) isolates from starch extraction stage, 16.7% (2/12) isolates from waste discharging stage and finally 8.3% (1/12) isolates from drying stage.

3.3.2 PFGE analysis

Twenty randomly chosen *B. cereus* isolates from sago processing A and B were subjected to PFGE analysis. The objective of PFGE analysis in this study was to group these bacteria isolates based on its genetic diversities according to sampling sites. Based on the gel photograph in Figure 3.11 and 3.12, the electrophoretic profile of DNA fragments obtained after PFGE for both sago processing mills yielded 1 - 24 bands with size approximately 10 kb to 1300 kb. The dendrogram shown in Figure 3.13 were constructed by using Bionumerics 7.6.2 software.

Based on the dendrogram constructed, *B. cereus* isolates were grouped into two major clusters; cluster A and cluster B. All isolates in cluster A belongs to sago processing mill A whereas all isolates from sago processing B were clustered in cluster B.

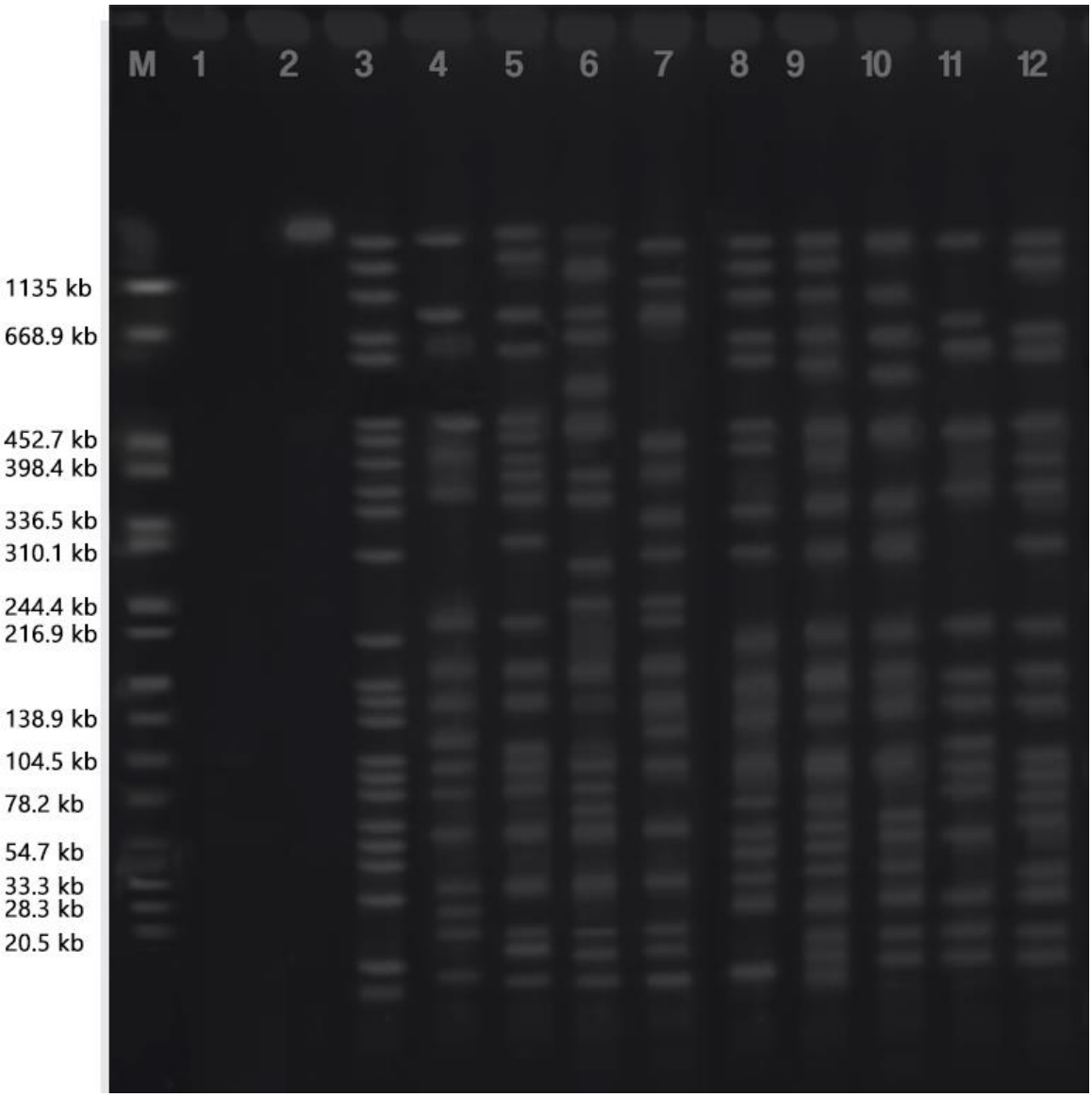

Figure 3.11: Banding profiles of PFGE for sago processing mill A. **Lane M:** DNA size marker, *Salmonella enterica* serotype *Braenderup* H9812. **3:** SPA 14, **4:** SPA 16, **5:** SPA 17, **6:** SPA 18, **7:** SPA22, **8:** SPA 28, **9:** SPA 29, **10:** SPA 64, **11**: SPA 65, **12:** SPA 69

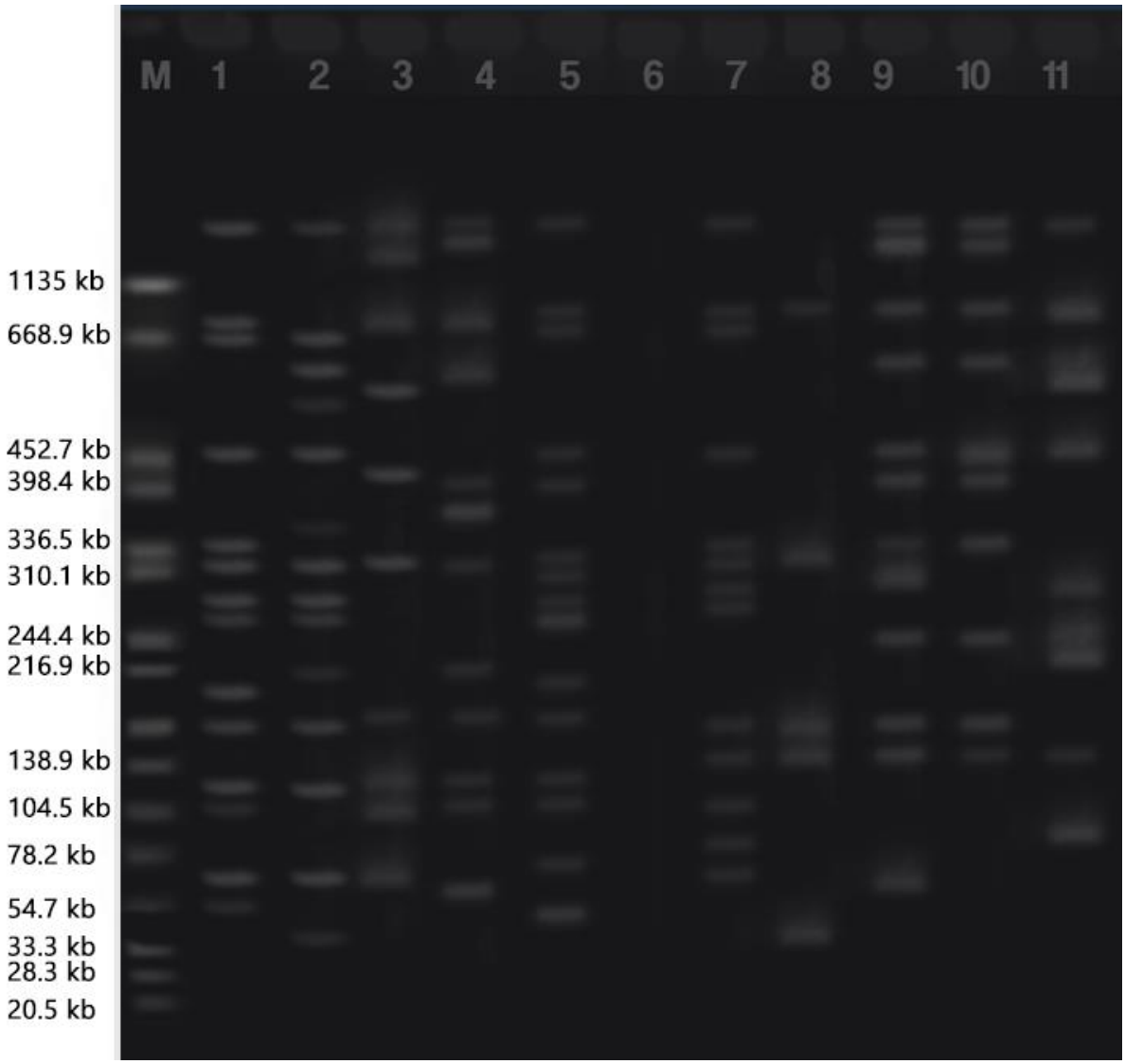

Figure 3.12 Banding profiles of PFGE for sago processing mill B. **Lane M:** DNA size marker, *Salmonella enterica* serotype *Braenderup* H9812. **1:** SPB 11, **2:** SPB 12, **3:** SPB 13, **4:** SPB 14, **5:** SPB 22, **7:** SPB 28, **8:** SPB 61, **9:** SPB 66, **10**: SPB 67

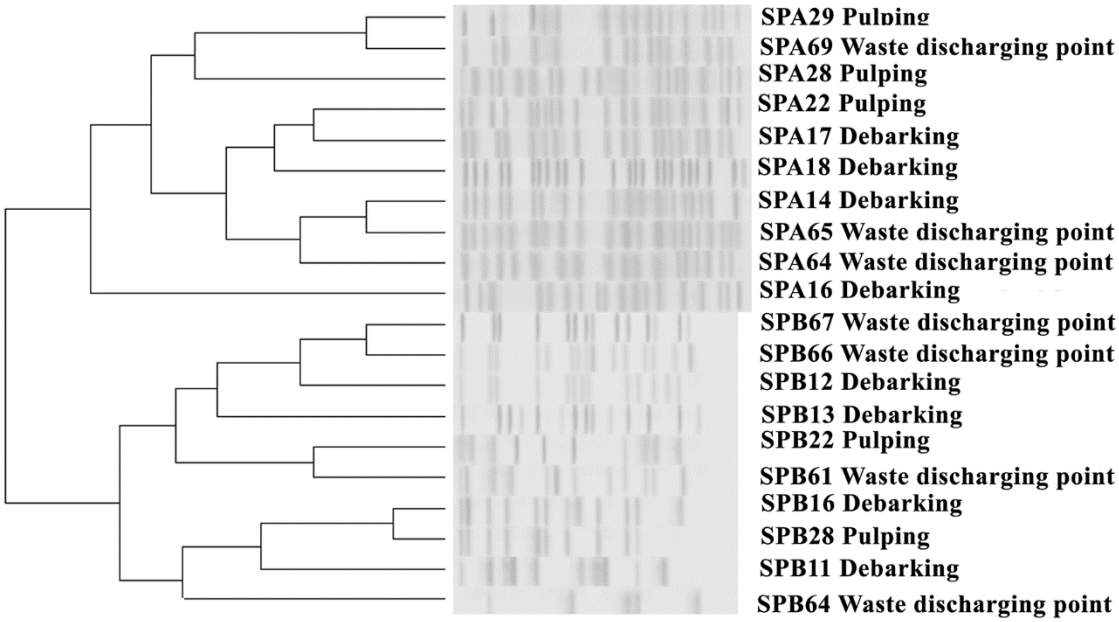

Figure 3.13: Dendrogram of PFGE profiles for sago processing mill

3.3.3 Antibiotic resistance, ERIC-PCR and PFGE pattern of *B. cereus* isolated from sago processing in Sarawak

For sago processing A, antibiotic resistance pattern were corresponded with ERIC-PCR and PFGE pattern whereas for sago processing B did not show any specific correspondation between antibiotic resistance, ERIC-PCR and PFGE pattern. The summarization of antibiotic resistance, ERIC-PCR and PFGE pattern of *B. cereus* was tabulated in Table 3.6.

Table 3.6: Antibiotic resistance and ERIC PCR patterns of *B. cereus* isolated from sago processing in Sarawak.

Location	Isolate	Processing step	Sample	Antibiotic Resistant Pattern	Antibiogram Pattern	ERIC-PCR Pattern	PFGE Pattern
SAGO PROCESSING MILL A	SPA 11	Debarking	Bark swab	DO/C/F/KF	1	1	NA
	SPA 12			DO/C/CRO/KF	2	2	NA
	SPA 14			DO/CRO/F/KF	3	3	1
	SPA 16			CRO/K/E/SXT	4	4	2
	SPA 17			C/CRO/F/KF/E	5	5	3
	SPA 18			DO/C/CRO/F/KF	6	6	4
	SPA 22	Pulping	Sago pith	DO/C/CRO/F/KF/ K/E/S/SXT	7	7	5
	SPA 28			DO/CRO/KF/K/E/S	8	8	6
	SPA 29			DO/C/KF/K/E/S	9	9	7
	SPA 64	Waste discharging	Sago effluent	DO/C/CRO/KF	10	10	8
	SPA 65			KF	11	11	9
	SPA 69			CRO/KF/TOB	12	12	10
SAGO PROCESSING MILL B	SPB 11	Debarking	Bark swab	DO/C/CRO/F/KF	4	12	11
	SPB 12			KF	11	13	12
	SPB 13			KF/SXT	13	13	13
	SPB 14			CRO/KF/E	14	14	14
	SPB 15			KF/K/S	15	15	NA
	SPB 16			F/KF/K/S	16	16	NA
	SPB 17			C/CRO/F/KF/E	6	17	NA
	SPB 18			C/CRO/F/KF	17	18	NA
	SPB 19			CRO/E	18	19	NA
	SPB 21	Pulping	Sago pith	DO/C/F/KF/K/E/S/ SXT	19	20	NA

Table 3.6 continued

	SPB 22			CRO/F	20	21	NA
	SPB 24			CRO/KF	21	22	15
	SPB 27			DO/C/CRO/F/KF	4	23	NA
	SPB 28			C/CRO/KF	22	24	16
	SPB 29			KF/TOB	23	25	NA
	SPB 31	Starch extraction	Starch slurry	C/CRO/SXT	24	26	NA
	SPB 35			C/KF/SXT	25	27	NA
	SPB 37			DO/C/KF/SXT	26	26	NA
	SPB 39			DO/C/KF	27	28	NA
	SPB 47	Drying	Sago milk	DO/KF	28	29	NA
	SPB 48			DO/C/F/KF	1	30	NA
	SPB 61	Waste discharging	Sago effluent	CRO/K/E/S/SXT	29	31	17
	SPB 62			CRO/F	20	31	NA
	SPB 63			DO/C/CRO/F/KF	4	28	NA
	SPB 64			DO/C/CRO/F/KF/K/E/S/SXT	7	18	NA
	SPB 65			DO/CRO/KF/K/E/S	30	32	NA
	SPB 66			C/KF/K/E/S	31	22	18
	SPB 67			DO/C/CRO/KF	10	33	19
	SPB 68			KF	11	34	NA
	SPB 69			CRO/KF/K	32	35	NA

NA = Not available

CHAPTER 4

DISCUSSION

4.1 Prevalence of *B. cereus* associated with sago processing in Sarawak

In this study, a total of 120 samples were collected from every stage of sago processing in sago processing A and sago processing B. The samples were consisted of bark swab (n=20), sago pith (n=20), starch slurry (n=20), sago milk (n=20), sago flour (n=20) and sago effluent (n=20) were collected from debarking, pulping, starch extraction, drying, packaging and waste discharging stage respectively. According to the best of our knowledge, present study is the first study of the detection of *B. cereus* in sago processing. However, *B. cereus* had been isolated from other food processing plant such as fresh-cut vegetable salad processing in Korea (Kim *et al.,* 2016), red pepper powder processing plant (Oh *et al.,* 2012), dairy plant (Svensson *et al.,* 2004) and chilled zucchini puree processing plant (Guinebrietre *et al.,* 2002). *B. cereus* had been isolated in sago starch in Papua New Guinea (Greenhill *et al.,* 2007). *B. cereus* were also isolated from varieties of food such rice grain (Lesley *et al.,* 2016), ready-to-eat vegetables (Chon *et al.,* 2015), Korean fermented soybean product (Kim *et al.,* 2015), ready-to-eat (RTE) in Sarawak, Malaysia (Lesley *et al.,* 2013), dairy products and processing plant in Brazil (Bernardes *et al.,* 2013), ready-to-eat rice in Malaysia (Sandra *et al.,* 2012), fresh vegetables and refrigerated minimally processed foods in Spain (Valero *et al.,* 2002), pasteurized milk (Eneroth *et al.,* 2001) and commercial cooked chilled foods containing vegetables in France (Choma *et al.,* 2000).

Studies on prevalence of *B. cereus* in different type of foods had been carried out by different researchers from other countries and Malaysia conveyed various result trend from the

present study. In the present study, it was revealed that 35% (42/120) of the samples from both sago processing mill A and sago processing mill B were positive with *B. cereus. B. cereus* was present in 20% (12/60) of the samples collected in sago processing mill A and 50% (30/60) of the samples collected in sago processing mill B. The prevalence and concentration of *B. cereus* in present study is comparable (35% - 45%) to the study conducted by Reyes *et al.* (2007) whereby in their study, the prevalence of *B. cereus* in dried milk product was 45% (175/381). The incidence of *B. cereus* in the products were fairly high, however the concentration of *B. cereus* is low. However, there were a higher prevalence of *B. cereus* had been isolated in rice grains (Lesley *et al.,* 2016) whereby 100% of the rice grains tested were found to have *B. cereus*. High prevalence also shown in a study by Sawei and Sani (2016) and Sandra *et al.* (2012), 100% and 93.33% of rice samples were positive for *B. cereus* respectively. It may due to the nature of *B. cereus* that are easily found in environment such as soil, dust, sediment and plants (Stenfors *et al.,* 2008). The prevalence of *B. cereus* in Korean fermented soy products (Kim *et al.,* 2015) also showed a higher prevalence whereby 67.9% of samples were detected with *B. cereus*. A study by Oh *et al.* (2012) on the prevalence of *B. cereus* along the processing stage of red chilli pepper processing plant 85% of *B. cereus* were detected and this also revealed a higher prevalence of *B. cereus* compared to present study. The prevalence of *B. cereus* in this study is lower as compared to the study conducted by Greenhill *et al.* (2007) which detected 74.4% (32/43) of *B. cereus* in sago starch in Papua New Guinea, Indonesia.

In contrast, the prevalence of *B. cereus* in fresh-cut vegetable salad processing (Kim *et al.,* 2016) is lower compared to the present study. In their study, 30.8% of *B. cereus* were detected along the processing line. Lesley *et al.* (2013) in their study also detected 13.3% of *B. cereus* in RTE cereal in Sarawak which is a lower prevalence of *B. cereus* compared to the present study. Similarly, low prevalence also revealed in RTE cereal in Korea (Park *et al.,* 2009)

which 25% of the samples were positive with *B. cereus.* Lower detection of *B. cereus* due the processing step reduced the *B. cereus* (Choma *et al.,* 2000), strict processing condition (Fang *et al.,* 2003) and continuous surveillance from the authorities (Wei *et al.,* 2006).

In this study, the prevalence and bacterial count in sago processing mill B is higher compared to sago processing mill A. For both sago processing mills, the sago logs were transported via rivers and were left unattended in the river for a while before being processed by the workers. This might contributes to the contamination of the sago logs with the bacteria. This is in agreement with the study by Muyibi *et al.* (2008) which stated that wastes from human and agricultural activities were the major source of river contamination in Malaysia. The sago logs were also observed to be left unattended on the soil surface and it might harbor *B. cereus*. *B. cereus* is commonly found in soil as it does not have complex nutrient requirement and it can spread easily (Kotiranta *et al.,* 2000).

Sago processing mill A located at a remote area, in the middle of the estate where there were little human activities and no human settlement observed nearby the processing mill and this explains the lower number of bacteria in the samples collected in sago processing mill A. However, the scenario in sago processing mill B was different. It was located in the middle of the village which was nearby the human settlement and agricultural area were also observed along the riverbank. Hence, human and agricultural activities were active nearby the sago processing mill A. Therefore, with many on-going activities by human, animals and agriculture, it is not surprising to obtain high prevalence and total bacterial count from sago processing mill B. In addition, *B. cereus* also has been reported to be present in human stools (Jensen *et al.,* 2003). This also explained the high concentration of *B. cereus* in sago processing mill B. Being present in human stools, it is expected that *B. cereus* was present in the environment such as in

the river due to higher human activities and human settlement along the river (Stenfors *et al.,* 2008).

From the comparison and observation with every bacterial count obtained from every processing mill, it was understood that the location of sago processing mills does affect the bacterial count in the population. It was in agreement with the research conducted by Alkaid *et al.* (2005). Their research suggest that the location of the farm affect the pathogen population. It was revealed that the further the location of farms from human settlements, animal contacts and disturbance, the lower the incidence of human pathogens in the farm. Ling *et al.* (2013) also reported that human settlements and agricultural activities along the rivers can be a potential threat to the water quality.

For both sago processing mills, towards the end product (sago flour in packaging stage) showing the decreasing trend of the prevalence and the bacterial count of *B. cereus*. It is due to the processing steps itself that allow the reduction of prevalence and bacterial count of *B. cereus*. It is comparable to the study conducted by Choma *et al.* (2000) which they revealed the number of *B. cereus* in processed vegetable was not more than 10 CFU/g where as in raw vegetable, the numbers of *B. cereus* was more than 10^2 CFU/g. They presumed the processing steps reduced the number of *B. cereus* in the end product. The application of wet heat at the drying stage also led to the reduced number of the prevalence and bacterial count of *B. cereus* and its spores. *B. cereus* is a mesophilic organism which grows between 10^oC to 50^oC with an optimum temperature of 35^oC to 40^oC (Lotte *et al.,* 2008) and can survive and produce spores up to 90^oC (Choma *et al.,* 2000). However, according to Coleman *et al.,* (2010), application of wet heat appears to kill *B. cereus* and its spores by denaturing one or more key proteins.

4.2 Antibiotic Susceptibitily Test

In antibiotic resistance analysis, the antibiotics contamination level reflects the antibiotic utilization in the studied area. The higher the frequency of resistant microorganism in certain environment, the higher the usage frequency of an antimicrobial agent in that environment (Samuel *et al.*, 2011). In present study, antibiotic susceptibility test were carried out using Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI). The antibiotic tested were doxycycline (30 µg), chloramphenicol (30 µg), ceftriazone (30 µg), norfloxazin (10 µg), cephalotin (10 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), kanamycin (30 µg), imipenem (10 µg), erythromycin (15 µg), tobramycin (10 µg), streptomycin (10 µg), sulphamethoxazole (30 µg), ampicillin (10 U) and penicillin (10 U). The antibiotic were selected following the study by Schlegelove *et al.* (2003) which were important in treating infectious disease in hospital (erythromycin, vancomycin, gentamycin, kanamycin, tobramycin, chloramphenicol, doxycycline, ceftriazone, cephalotin) treatment of bovine mastitis on farm (ampicillin, cephalotine, clindamycin, neomycin, oxacillin, tetracycline and streptomycin), drugs that under regulation limiting the usage of selected antibiotics in the treatment of animal (norfloxacin). It was revealed that *B. cereus* in the present study has highest resistance towards penicillin (100%) and ampicillin (100%).

It is in close agreement with the research by Park *et al.* (2009). In their study on antibiotic resistance toward *B. cereus* in rice and cereals in Korea, all the isolates (100%) were highly resistant towards ampicillin and penicillin. High ampicillin- and penicillin-resistance were also recorded by Coonrod (1971), where by all the clinical samples (100%) were observed to be resistant towards ampicillin and penicillin. On the other hand, *B. cereus* from food samples in the research conducted by Schlegelova *et al*. (2003) were also observed to be resistant towards ampicillin. Similarly, Rusul *et al.* (1995) observed that *B. cereus* isolated from selected food were highly resistant (98.8%) towards ampicillin. Andrew *et al.* (2002) also reported high

resistance (100%) of penicillin towards *B. cereus* isolates in their diagnostic lab. It is in close agreement with Weber *et al.* (1988) whereby all the *B. cereus* from blood culture were resistant towards penicillin.

High resistance towards ampicillin and penicillin were not surprising. It is because there were among the first antibiotics introduced (Tortora *et al.,* 2007). High resistance towards penicillin, ampicillin and other ß-lactam were influenced by the production of β-lactamases enzyme in *B. cereus* (Chen *et al*., 2004; Kotiranta *et al*., 2000). The enzyme deactivate the antibacterial properties molecules by breaking the ring open and cause the bacteria to be resistance towards the β-lactam antibiotic such as penicillin and ampicillin (Jalalpoor *et al*., 2012). There are three form of β-lactamases in *B. cereus* strains and one of it is penicillinase. Penicillase is a specific enzyme that hydrolyze the penicillin antibiotic (Kotiranta *et al.,* 2000). According to Coonrod (1971), *B. cereus* produce penicillinase and responsible for the penicillin resistance. In addition, the production of crystalline layer by *B. cereus* protect the bacteria from the influence of antibiotic and harmful enzymes (Jalalpoor *et al.,* 2010). High percentage of susceptibility was observed on antibiotics imipenem (100%), norflaxozin (100%) and tobramycin (96%). It is in close agreement to a study by Chon *et al*. (2012), *Park et al.* (2009), Andrews *et al*. (2002) and Weber *et al.* (1998). It is not surprising since imipenem could be prescribed by doctors as a treatment for bacterial infection (Letchumanan *et al.,* 2015). Hence, the finding of the antibiotic resistance of *B. cereus* isolates in sago processing are therefore consistent with the earlier findings.

In this study, 76.1% (32/42) isolates have MAR index more than 0.2 which are 91.7% (11/12) and 70% (21/30) from sago processing A and B respectively. Multiple antibiotic resistant bacteria might have travelled through water. It is in agreement with Buschman *et al.* (2012) where by the antibiotic resistant bacteria transported by water current. In addition,

Gelband *et al.* (2015) state that multiple antibiotic resistant travel locally and globally. The occurrence of antibiotic resistance also affected by few factors such as stress due to temperature and hence lead to the selection of antibiotic resistance bacteria (Samuel *et al.,* 2011). The selection of antibiotics resistance bacteria cause an increased risk of resistant pathogens to be transferred to human, thus may pose public health risk (Chon *et al.,* 2015). The usage of animal wastes as the fertilizer that usually contain antibiotic also lead to MAR of *B. cereus* in sago processing. Antibiotics are commonly used to treat animal disease and as growth hormone in agriculture. Hu *et al.* (2010) in their study on the migration of antibiotics from manure to soil and from soil to plant showed that antibiotic residues were detected in soil, plants and water in the vegetable farm that applied manure containing antibiotics. Hence, this proved that antibiotics were able to migrate from manure to soil and vegetables. This situation poses great risk on the safety of ecosystems and human health.

Apart from that, the present study suggest that the environment of sago plantation, the transmission process to the sago processing mills and sago processing mills itself might exposed to the heavy metals present in polluted external environment might enrich the MAR bacteria. It is explained in a study conducted by Gullberg *et al.* (2014) which revealed that low levels of heavy metals present in polluted external environments could allow selection and enrichment of multidrug resistance bacteria. Thus, it contributes to the emergence, maintenance, and transmission of antibiotic-resistant disease-causing bacteria.

The percentage of the susceptibilities and resistance of certain in present study are varies. It may due to the different concentration of antimicrobial agents used, different sources of isolates, drug resistance transfer and the wide spread use of the antibiotics in the environment (Whong *et al.,* 2007). In present study, the antibiotic resistance pattern were highly variable and most of the isolates were resistance to multiple antibiotics. The multiple resistant patterns in

this study might reflect that the antibiotics were highly abused in this environment. The study on the pattern is important for epidemiological studies of *B. cereus*. However, the comparison of the present AST results with previous works are not possible because there was no previous AST data of sago processing in Sarawak.

4.3 Genotyping of *B. cereus* associated with sago processing in Sarawak

In present study, ERIC-PCR and PFGE were used to study the relatedness of the *B. cereus* isolates from different type of samples and sampling areas. Dendrograms were constructed to show the relationships between identical strains and also similar groups of strains. Isolates in the same lineage are usually found to be genetically related and/or indicate possible cross-contamination (Shi *et al.,* 2015). In this study, ERIC-PCR yielded 1 - 7 bands with size approximately 100 bp to 990 bp. From both sago processing mills, 35 DNA fingerprint profile were shown. The findings in the present study showed that the *B. cereus* isolates were not group together based on the types of samples and the source of isolation. It is in agreement with a study by Merzougui *et al.* (2013), they isolated *B. cereus* from different food samples such as rice salads, dairy products, milk and spices and they were not classified into specific cluster neither by sampling area nor the type of samples. The present study is also comparable to a study conducted by Kim *et al*. (2014), whereby *B. cereus* isolated from fermented soy bean products showed high genetic diversity and the banding pattern of rep-PCR were not associated with specific product types. This indicate the high heterogeneity and diversity of *B. cereus* strain in both sampling sites. In the present study, the *B. cereus* isolated from each sago processing mills were genetically diverse and heterogeneous. The heterogeneity was expected as the isolates were collected from different types samples. It may also due to the ubiquitous nature of *B. cereus* where there are easily found in different type of environment (Swiecicka, 2008). Hence, the contamination can occur from different sources such as soil, water, tree bark and

sago processing mills equipment. However, in a study done by Kim *et al.* (2016), they isolated *B. cereus* from fresh cut vegetables and molecular typing of *B. cereus* were conducted by rep-PCR. In their study, 13 samples of fresh cut vegetables from company A where 10 samples were obtained at retail level of company A and another 3 were obtained from the processing line of company A. It was revealed that 11 from 13 isolates tested were group into 3 clusters which is comprised of two or more isolates with a similarity more than 95%. This indicates the low diversity of *B. cereus* and the same clone of *B. cereus* have been circulated in the company A and they suggested that the contamination might came from the raw materials of the product. The findings of the study by Kim *et al*. (2016) also show a similar trend to a study by Oh *et al.* (2012) where they isolated and performed molecular typing by rep-PCR in red pepper powder processing plant. High genetic similarity of *B. cereus* in their finding suggested that all the isolates may have originated from the same clone.

In present study, PFGE analysis were conducted to twenty randomly chosen *B. cereus* isolates from sago processing A and B. Based on the gel photograph in Figure 3.6 and 3.7, the electrophoretic profile of DNA fragments obtained after PFGE for both sago processing mills yielded 1 - 24 bands with size approximately 10 kb to 1300 kb. Based on the dendrogram constructed, *B. cereus* isolates were grouped into two major clusters; cluster A and cluster B. All isolates were grouped based on sampling sites, which is in cluster A belongs to sago processing mill A whereas all isolates from sago processing B were grouped in cluster B. It is comparable to a study by Merzougui *et al.* (2013). Their study revealed that there were visible correlation between the PFGE DNA fingerprint profiles and sources of isolates.

From both molecular typing methods, both typing results revealed inconsistency. Some strains showed concordant typing results in PFGE and ERIC-PCR and some strains had diverging results. It is because, even though both PFGE and ERIC-PCR were to test the genetic

diversity, both of the methods were not equivalent and worked in different ways (Merzougui *et al.,* 2013). PFGE is a whole-genome characterization method and reveals restriction site polymorphisms arising from the genomic locations of restriction endonucleases sites (Zhong *et al.,* 2007) and ERIC-PCR reveals a profile of DNA fragments of different sizes based, in principle, on the genomic locations of specific repetitive sequences (Li *et al.,* 2011). Even though PFGE is a gold standard for molecular typing, it is laborious, time-consuming and limited number of samples can be tested (Merzougui *et al.,* 2013) compared to Rep-PCR such as ERIC-PCR is reproducible, sensitive, fast and cheap. It has proven that it is rapid and reliable method that has been successfully applied to bacterial typing (Oh *et al.,* 2012). Therefore, rapidness, performance easiness, the ability to differentiate between closely related strains and reproducibility are among the useful criteria for better genotyping method (Samuel *et al.,* 2011).

In present study, it reveals that there is the potential of the domination of single strain of *B. cereus* circulating in sago processing mills. However it is also reveals that there is also potential of different strain of diverse genetic contents also circulated in sago processing mill. However, some of the cluster showed that they were classified into specific cluster by stages of samples were collected. It revealed that high prevalence of similar strain of *B. cereus* and it had become dominant and better adapted in those specific processing i.e. Debarking step of sago processing mill B. The findings of present study was meaningful and should be considered because it suggest that cross-contamination occurred between the processing steps in sago processing mills. Cross-contamination could lead to serious foodborne illness which is a widespread health problem that create social and economic burden as well as human suffering. Therefore, strict hygiene and good management and manufacturing practices of sago processing must be adopted to avoid unwanted illness due to consumption of contaminated sago and sago products.

Results of the current study can be used as baseline data in risk estimation for the prediction of human illness associated with raw sago processing and provide useful information for the microbial risk assessment of *B. cereus*. The bacterial isolates observed in this study are suspected to contaminate the sample from various sources, which could be due to poor handling and storage after tree felling. The environment, utensils used, the state of environmental hygiene of the sago processing mills were all possible source of contamination. It is recommended that the sago processing should be done with utmost hygienic measure and that bark immediately processed after collection to reduce the load of bacteria especially the pathogenic ones. Hygiene measures presume a decisive importance in food safety management (Akanele *et al.*, 2016).

CHAPTER 5

CONCLUSION

This study provides an overview and baseline information of the prevalence and occurrence of *B. cereus* in sago processing mill A and sago processing mill B in Sarawak, Malaysia. The finding showed that the prevalence of *B. cereus* in this study was 35%. It was also revealed that sago processing B had higher occurrence of *B. cereus* when compared to sago processing A. As discussed earlier, the location factors and manufacturing practices in sago processing B, which was located in human settlement and agricultural area. It this study, it was discovered that the prevalence of *B. cereus* were reduced towards the end products. It was suggested that the processing itself reduce the prevalence and contamination of *B. cereus*.

In this research, molecular typing by using ERIC-PCR and PFGE were also conducted. It revealed that the isolates were heterogeneous as they were not classified into specific cluster neither by sampling area nor the type of samples. Isolates in the same lineage are usually found to be genetically related and/or indicate possible cross-contamination. Antibiotic resistance profile of *B. cereus* was also determined in present study. In present study, most of the *B. cereus* isolates were resistant towards ampicillin and penicillin and susceptible towards norfloxazin and imipenem. Among 42 isolates tested, 100% (42/42) which were against penicillin and ampicillin, followed by cephalotin 88.1% (37/42), ceftriazone 61.9% (26/42), chloramphenicol 52.4% (22/42), doxycycline 45.2% (19/42), nitrofurantoin 35.7% (15/42), kanamycin 28.6% (12/42), streptomycin 28.6% (12/42), sulphamethoxazole 19.0% (8/42), tobramycin 4.8% (2/42), imipenem 0.0% (0/42) and norfloxazin 0.0% (0/42).

The MAR index for all isolates were also varies, ranged from 0.083 to 0.750. MAR index higher than 0.20 indicates that the particular organism is originated from high-risk sources

of contamination, where antibiotics are often used. MAR index less than or equal to 0.20, indicates that the isolate is originated from environment where antibiotics are seldom or never used. The findings of present study were meaningful and should be considered because outbreaks of *B. cereus* emetic food poisoning are usually linked to starchy products. This study suggest that cross-contamination occurred between the processing steps in sago processing mills. Cross-contamination could lead to serious foodborne illness which is a widespread health problem that create social and economic burden as well as human suffering. Therefore, strict hygiene and good management and manufacturing practices of sago processing must be adopted to avoid unwanted illness due to consumption of contaminated sago and sago products. The bacterial isolates observed in this study are suspected to contaminate the sample from various sources, which could be due to poor handling and storage after tree felling. The environment, utensils used, the state of environmental hygiene of the sago processing mills were all possible source of contamination. It is recommended that the sago processing should be done with utmost hygienic measure and that sago barks are cleaned and immediately processed after collection to reduce the load of bacteria especially the pathogenic ones. Hygiene measures presume a decisive importance in food safety management.

The findings in this study provides baseline data on *B. cereus* contamination at farm level for future risk assessment work and for establishing food safety standards in sago processing in Sarawak, Malaysia. Therefore, further study on the risk assessment and microbial biofilm of *B. cereus* on abiotic surfaces especially in sago processing environment can be conducted in the future.

REFERENCES

Acai, P., Valík, L., & Liptáková, D. (2014). Quantitative risk assessment of *Bacillus cereus* in pasteurised milk produced in the Slovak Republic. *Czech Journal of Food Sciences*, *32*(2), 122–131.

Adeni, D. S., Aziz, S., Bujang, K., & Hassan, M. A. (2009). Bioconversion of sago residue into value added products. *African Journal of Biotechnology*, *9*(14), 2016–2021.

Agne, M., Valones, A., Guimarães, R. L., André, L., & Brandão, C. (2009). Principles and application of polymerase chain reaction in medical diagnostic fields: A review. *Brazilian Journal of Microbiology*, *40*, 1–11.

Akanele, E., Mgbo Otu Chukwu, S., & Mary Ahudie, C. (2016). Microbiological contamination of food: The mechanisms, impacts and prevention. *International Journal of Scientific & Technology Research*, *5*(3), 65-78.

Alkaid, E., Blaco, M.D. & Esteve, C. (2005). Occurrence of drug-resistant bacteria in two European eel farms. *Applied and Environmental Microbiology*, *71*, 3348-3350.

Aminov, R. I. (2010). A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology*, *1*(Dec), 1–7.

Andrews, J. M. (2002). Susceptibility testing of *Bacillus* species. *Journal of Antimicrobial Chemotherapy*, *49*(6), 1039–1046.

Anita, T., Singh, S. P., & Rashmi, S. (2012). Prevalence of multidrug resistant *Bacillus cereus* in foods and human stool samples in and around Pantnagar, Uttrakhand. *Journal of Advanced Veterinary Research*, *2*, 252–255.

Banerjee, M., Nair, G. B., & Ramamurthy, T. (2011). Phenotypic & genetic characterization of *Bacillus cereus* isolated from the acute diarrhoeal patients. *Indian Journal of Medical Research*, *133*(1), 88–95.

Basim, E. (2001). Pulsed-Field Gel Electrophoresis (PFGE) technique and its use in molecular biology. *Turkey Journal Biology*, *25*(2), 405-418.

Bernardes, P. C., Andrade, N. J. De, Penã, W. E. L., Minim, L. A., Sá, J. P. N., Fernandes, P. É., & Colombari, D. D. S. (2013). Modeling of the adhesion of *Bacillus cereus* isolated from a dairy plant as a function of time and temperature. *Journal of Food Process Engineering*, *36*(2), 187–191.

Byarugaba, D. K. (2010). *Mechanisms of antimicrobial resistance*. *Antimicrobial Resistance in Developing Countries* (pp. 15–26). New York, NY: Springer New York.

Bujang, K. (2008). Potentials of bioenergy from the sago industries in Malaysia. *Biotechnology: Encyclopedia of Life Support Systems*. Brisbane, Australia.

Bujang, K. (2010). Production and processing of sago: Food and fuel alternative. Paper presented in International Seminar on Sago & Spices for Food Security. Ambon. Indonesia. Retrieved from https://www.researchgate.net/publication/263351072_sago_a_food_and_fuel_alternative

Bujang, K. & Muniandy, D. (2004). Production of soil conditioner from sago hampas. Expo Malaysian Science and Tchnology Exhibition. COSTAM. The Palace of Golden Horses, The Mines, Kuala Lumpur, Malaysia. 5-7th October, 2004.

Buschmann, A. H., Tomova, A., Lopez, A., Maldonado, M. A., Hendriques, L. A., Ivanova, L., Moy, F., Godfrey, H. P. & Cabello, F. C. (2012). Salmon aquaculture and antimicrobial resistance in marine environment. *PLoS ONE, 7(*8), 1-11.

Centers for Disease Control and Prevention (CDC). (2013). Surveillance for foodborne disease outbreaks — United States, 2009–2010. *United States of America.* Retrieved December 26, 2016, from https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6203a1.htm.

Centers for Disease Control and Prevention (CDC). (2005). Outbreak of cutaneous *Bacillus cereus* infections among cadets in a university military program --- Georgia, August 2004. *United States of America.* Retrieved December 26, 2016, from https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5448a3.htm

Chang, H.J., Lee, J.H., Han, B.R., Kwak, T. K., & Kim, J. (2011). Prevalence of the levels of *Bacillus cereus* in fried rice dishes and its exposure assessment from Chinese-style restaurants. *Food Science and Biotechnology*, *20*(5), 1351-1359.

Chen, Y., Chen, Y., Tenover, F. C., Tenover, F. C., Koehler, T. M., & Koehler, T. M. (2004). Beta-Lactamase Gene Expression in a Penicillin-Resistant. *Society*, *48*(12), 4873–4877.

Choma, C., Guinebretière, M. H., Carlin, F., Schmitt, P., Velge, P., Granum, P. E., & Nguyen-The, C. (2000). Prevalence, characterization and growth of *Bacillus cereus* in commercial cooked chilled foods containing vegetables. *Journal of Applied Microbiology*, *88*(4), 617–625

Chon, J. W., Yim, J. H., Kim, H.-S., Kim, D. H., Kim, H., Oh, D. H. & Seo, K. H. (2015). Quantitative prevalence and toxin gene profile of *Bacillus cereus* from ready-to-eat vegetables in South Korea. *Foodborne Pathogens and Disease*, *12*(9), 795–799.

Coleman, W. H., Zhang, P., Li, Y. Q., & Setlow, P. (2010). Mechanism of killing of spores of *Bacillus cereus* and *Bacillus megaterium* by wet heat. *Letters in Applied Microbiology*, *50*(5), 507–514.

Coonrod, J. D., Leadley, P. J., & Eickhoff, T. C. (1971). Antibiotic susceptibility of *Bacillus* species. *The Journal of Infectious Diseases*, *123*(1), 102–105.

Das, S. & Dash, H. R. (2015). Basic molecular biology of bacteria: Isolation of genomic DNA. Microbial biotechnology - A laboratory manual for bacterial systems. Springer India. Retrieved July 31, 2015, from https://books.google.com.my/books/

Department of Agriculture Sarawak. (2013). Sarawak Agriculture Statistics 2013. Malaysia. Retrieved January 2, 2017, from http://www.doa.sarawak.gov.my/

Dierick, K., Coillie, E. Van, Swiecicka, I., Devlieger, H., Meulemans, A., Fourie, L., & Mahillon, J. (2005). Fatal family outbreak of *Bacillus cereus* - Associated food poisoning fatal family outbreak of *Bacillus cereus* - Associated food poisoning. *Journal of Clinical Microbiology*, *43*(8), 4277–4279.

Doyle, M. (1989). Foodborne Bacterial Pathogens. Taylor & Francis. Retrieved from https://books.google.com.my/books?id=qbAguo2yYuAC

Ehara, H., Susanto, S., Mizota, C., Hirose, S., & Matsuno, T. (2000). Sago palm (*Metroxylon sagu*, Arecaceae) production in the eastern archipelago of Indonesia: Variation in morphological characteristics and pith dry-matter yield. *Economic Botancy*, *54*(2), 197–206.

Ehling-schulz, M., Fricker, M., & Scherer, S. (2004). Review *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. *Molecular Nutritional Food Research*, *48*(2), 479–487.

Eneroth, A., Svensson, B., Molin, G., & Christiansson, A. (2001) Contamination of pasteurized milk by *Bacillus cereus* in the filling machine. *Journal of Dairy Research*, *68*(1), 189–196.

Evans, A. S., & Brachman, P. S. (2013). *Bacterial Infections of Humans: Epidemiology and Control*. Springer US. Retrieved from https://books.google.com.my/books?id=dPDdBgAAQBAJ

Fakruddin, M., Mannan, K. S. B., Mazumdar, R. M., Chowdhury, A. & Hossain, M. N. (2013). Identification and characterization of microorganisms: DNA-fingerprinting methods. *Songklanakarin Journal of Science and Technology, 35*(4), 397-404.

Fallah, A. A., Saei-Dehkordi, S. S., & Mahzounieh, M. (2013). Occurrence and antibiotic resistance profiles of *Listeria monocytogenes* isolated from seafood products and market and processing environments in Iran. *Food Control, 34*(2), 630–636.

Fang, T. J., Wei, Q. K., Liao, C. W., Hung, M. J., & Wang, T. H. (2003). Microbiological quality of 18ºC ready-to-eat food products sold in Taiwan. *International Journal of Food Microbiology, 80*(2), 241– 250.

Food and Drug Administration (FDA). (2012) Bacteriological Analytical Manual: Chapter 14 *Bacillus cereus*. Retrieved January 2, 2017, from http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070875.htm

Food Safety News. (2016). Bacteria named in Mighty Taco outbreak; chain denies fault. *United States of America.* Retrieved December 26, 2016, from http://www.foodsafetynews.com/2016/10/bacteria-named-in-mighty-taco-outbreak-chain-denies-fault/#.WF_8RPl97IU.

Food Standards Australia New Zealand. (2001). Guidelines for the microbiological examination of ready- to-eat foods. Australia. Retrieved January 2, 2017, from https://www.foodstandards.gov.au/code/microbiollimits/documents/Guidelines%20for%20Micro%20exam.pdf

Frankland, G. C., Frankland, P. F., & Lankester, E.R. (1887). Studies on some new microorganisms obtained from air. *Philosophical Transaction of the Royal Society of London, 178*, 257-287.

Fukushima, H., Tsunomori, Y., & Seki, R. (2003). Duplex real-time SYBR Green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. *Journal of Clinical Microbiology, 41*(11), 5134–5146.

Gelband, H. (2015). The state of the world's antibiotics. *Wound Healing Southern Africa*, *8*(2), 30–34.

Garibyan, L. & Avashia, N. (2013). Research Techniques Made Simple: Polymerase Chain Reaction (PCR). *Journal of Investigation Dermatology, 133*(3), 1-8.

Ghelardi, E., Celandroni, F., Salvetti, S., Barsotti, C., Baggiani, A., & Senesi, S. (2002). Identification and characterization of toxigenic *Bacillus cereus* isolates responsible for two food-poisoning outbreaks. *FEMS Microbiology Letters*, *208*(1), 129–134.

Granum, P. E., & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters, 157,* 223–228.

Greenhill A.R., Shipton, W. A., Blaney, B. J., Amoa, B., Kopel, E., Pelowa, D. & Warner, J. M. (2007). Bacterial contamination of sago starch in Papua New Guinea. *Journal of Food Protection*, *70*(12), 2868–2872.

Greenhill, A. R., Shipton, W. A., Blaney, B. J., Brock, I. J., Kupz, A., & Warner, J. M. (2009). Spontaneous fermentation of traditional sago starch in Papua New Guinea. *Food Microbiology, 26*(2), 136–141.

Greenhill, A. R., Shipton, W. A., Blaney, B. J., Amoa, B., Kopel, E., Pelowa, D. & Warner, J. M. (2010). Hazards and critical control points for traditional sago starch production in Papua New Guinea: Implications for food safety education. *Food Control*, *21*(5), 657–662.

Guinebretiere, M., Broussolle, Y., & Nguyene, C. (2002) Enterotoxigenic profiles and food poisoning and foodbome *Bacillus cereus* strains. *Journal of Clinical Microbiology, 40*(2), 3053-3056.

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L., & Andersson, D. I. (2014). Selection of a multidrug resistance plasmid by sub-lethal levels of antibiotics and heavy metals. *American Society for Microbiology*, 5 (5), 1-9.

Hendriksen, N. B. (2001). Detection of Enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Applied and Environmental Microbiology*, 67(1), 185–189.

Hora, S. and Ali, Z. (2017). A study of relationship between antibiotic resistance and molecular characteristics of *Escherichia coli* isolates obtained from different human clinical specimens against multiple antibiotic resistance (MAR) index in Bareilly (India) region. *International Journal Pharmaceutical and Science Resources, 3(*9), 3331-3336.

Hu, X., Zhou, Q., & Luo, Y. (2010). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environmental Pollution*, *158*, 2992-2998.

Jalalpoor, S., Kasra, K. R., Noohi, A., & Zarkesh, H. (2010). Survey characterization nano structure surface layer in some of pathogen bacteria. *Journal of Resource Medical Science*, *12*(4), 3-10.

Jalalpour, S., & Ebadi, A. G. (2012). Role of nano structure of crystalline layer and beta lactamase nano enzyme in antibiotics resistant bacteria. *African Journal of Pharmacy and Pharmacology*, *6*(2), 113–118.

Jensen, G. B., Hansen, B.M., Eilenberg J., & Mahillon J. (2003). The hidden lifestyles of *Bacillus cereus* and relatives. *Environmental Microbiology, 5*, 631–640.

Julin, D. (2012). Polymerase Chain Reaction. In E. Bell (Ed.), *Molecular Life Sciences: An Encyclopedic Reference* (pp. 1–3). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4614-6436-5_95-4

Karim, A. A., Tie, A. P., Manan, D. M. A., & Zaidul, I. S. M. (2008). Starch from the sago (*Metroxylon sagu*) palm tree — Properties , prospects, and challenges as a new industrial source for food and other uses. *CRFSFS: Comprehensive Reviews in Food Science and Food Safety*, *7*, 215-228.

Kim, C. W., Cho, S. H., Kang, S. H., Park, Y. B., Yoon, M. H., Lee, J. B., & Kim, J. B. (2015). Prevalence, Genetic Diversity, and Antibiotic Resistance of *Bacillus cereus* Isolated from Korean Fermented Soybean Products. *Journal of Food Science, 80(1)*, 123–128.

Kim, H. J., Koo, M., Hwang, D., Choi, J. H., Kim, S. M., & Oh, S. W. (2016). Contamination patterns and molecular typing of *Bacillus cereus* in fresh-cut vegetable salad processing. *Applied Biological Chemistry*, *59*(4), 573–577.

Kim, J., Jeong, H., Park, Y., Kim, J., & Oh, D. (2010). Food poisoning associated with emetic-type of *Bacillus cereus* in Korea. *Foodborne Pathogen and Diseases*, *7*(5), 555-563.

Kim, Y.R. & Carl A. B. (2008). Riboprint and virulence gene patterns for *Bacillus cereus* and related species. *Journal of Microbiology and Biotechnology, 18*(6), 1146–1155.

Kotiranta, A., Lounatmaa, K., & Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes and Infection*, *2*(2), 189–198.

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied and Environmental Microbiology*, *46*(1), 165–170.

Lampel K.A., Orlandi P.A., & Kornegay L. (2000). Improved template preparations for PCR-based assays for detection of food-borne bacterial pathogens. *Applied Environment Microbiology*, *66(2)*, 4539-4542.

Lampel K.A., Al-Khaldi S., & Cahill S.M. (2012): Bad Bug Book. Foodborne pathogenic microorganisms and natural toxins handbook, 2nd Edition. Food and Drug Administration (FDA), Silver Spring.

Lee, N., Sun, J. M., Kwon, K. Y., Kim, H. J., Koo, M., & Chun, H. S. (2012). Genetic diversity, antimicrobial resistance, and toxigenic profiles of *Bacillus cereus* strains isolated from Sunsik. *Journal of Food Protection*, *75*(2), 225–30.

Lee, P. Y., Costumbrado, J., Hsu, C.-Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments*, *62*, 1-5.

Lesley, M. B., Velnetti, L., Yousr, A. N., Kasing, A., & Samuel, L. (2013). Presence of *Bacillus cereus* s.l. From ready-to-eat cereals (RTE) products in Sarawak. *International Food Research Journal*, *20*(2), 1031–1034.

Lesley, M. B., Tahar, A. S., Shze, T. P., Valarie, S., Anak, F., Hashim, H. F., & Radu, S. (2016). Enumeration and molecular detection of *Bacillus cereus* in local indigenous and imported rice grains. *Agriculture & Food Security*, 25(5), 4–8.

Letchumanan, V., Pusparajah, P., Tan, L. T. H., Yin, W. F., Lee, L. H., & Chan, K. G. (2015). Occurrence and antibiotic resistance of *Vibrio parahaemolyticus* from shellfish in Selangor, Malaysia. *Frontiers in Microbiology*, 6-1417.

Li, H., Liu, D., & Gao, J. (2011). Differentiation between *Bacillus thuringiensis* and *Bacillus cereus* by 16S rDNA-PCR and ERIC-PCR. *Journal of Northeast Agricultural University* (English Edition), *18*(3), 12–15.

Lin, T. H., Lin, L. Y. & Zhang, F. (2014). Review on molecular typing methods of pathogens. *Open Journal of Medical Microbiology, 4*, 147-152.

Ling, T.Y., Lim, S. W., Lesley M. B., & Lee, N. (2013). Impact of different land uses on *Escherichia coli* concentrations, physical and chemical water quality parameters in tropical stream. *Borneo Journal of Resource Science and Technology*, *2*(2), 42-51.

Liyanage, G. Y. and Manage, P. M. (2017). Determination of Multiple Antibiotic Resistant (MAR) and MAR Index in bacteria isolated from aquaculture farms. Paper presented in International Research Symposium of Pure and Applied Sciences.University of Kelaniya. Sri Lanka. Retrieved from http://repository.kln.ac.lk/bitstream/handle/123456789/18235/144.pdf?sequence=1&isAllowed=y

Lund, T., Buyser, M. De, Einar, P., & Aliments, Â. S. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology*, *38*(2), 254–261.

Malorny, B., Lofstrom, C., Wagner, M., Kramer, N. & Hoorfar, J. (2008). Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. *Applied and Environmental Microbiology, 74* (5), 1299– 1304.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J.S., Shapiro, C., Griffin, P. M., & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Disease 5*(5), 607-625.

Merzougui, S., Lkhider, M., Grosset, N., Gautier, M., & Cohen, N. (2013). Differentiation by molecular typing of *Bacillus Cereus* Isolates from Food in Morocco : PFGE-ERIC-PCR. *Food and Public Health, 3*(4), 223–227.

Michael, L. M. D., John, P. D., Rachel, W. H., & Christopher, B.M. (2006). Surveillance for foodborne disease outbreaks in United States, 1998-2002. *Centre of Disease Control, 55*(10), 1-34.

Mutashar, K., Aboodi, A. H., & Darweesh, M. J. (2015). Evaluation of diagnostic polymerase chain reaction (PCR) for the detection of *Escherichia coli* , *Staphylococcus aureus* and *Bacillus cereus* in cheese. *African Journal of Microbiology Research*, *9*(9), 611–616.

Muyibi, S.A., Ambali, A.R. & Eissa, G.S. (2008). The impact of economic development on water pollution: trends and policy actions in Malaysia. *Water Resource Managemant, 22*, 485-508.

Samuel, L., Marian, M. M., Apun, K., Lesley, M. B. & Son, R. (2011). Characterization of *Escherichia coli* isolated from cultured catfish by antibiotic resistance and RAPD analysis. *International Food Research Journal*, *18*(3), 971–976.

Oh, S. W., Koo, M., & Kim, H. J. (2012). Contamination patterns and molecular typing *of Bacillus cereus* in red pepper powder processing. *Journal of the Korean Society for Applied Biological Chemistry*, *55*(1), 127–131.

Omoloso, A. D. (1999). Microbial profile of sago starch from different areas of Papua New Guinea, p. 85–90. In P. A. Sopade (Ed.), Sago starch and food security in Papua New Guinea. *Proceedings of the First National Sago Conference*, 15 to 16 November 1999. Unitech Press, Lae, Papua New Guinea.

Onsa, G. H., Saari, N. Bin, Selamat, J., & Bakar, J. (2004). Purification and characterization of membrane-bound peroxidases from *Metroxylon sagu*. *Food Chemistry*, *85*(3), 365–376.

Osundiya, O. O., Oladele, R. O., & Oduyebo, O. O. (2013). Multiple antibiotic resistance (MAR) indices of *Pseudomona*s and *Klebsiella* species isolates in Lagos University Teaching Hospital. *African Journal of Clinical and Experimental Microbiology*, *14*(3), 164–168.

Owusu-kwarteng, J., Wuni, A., Akabanda, F., Tano-debrah, K., & Jespersen, L. (2017). Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus sensu lato* isolated from dairy farms and traditional dairy products. *BMC Microbiology*, *17*(1), 9–16.

Park, Y. B., Kim, J. B., Shin, S. W., Kim, J. C., Cho, S. H., Lee, B. K., & Oh, D. H. (2009). Prevalence, genetic diversity, and antibiotic susceptibility of *Bacillus cereus* strains isolated from rice and cereals collected in Korea. *Journal of Food Protection*, *72*(3), 612–7.

Pasic, M., Hojilla, C., & Yousef, G. M. (2014). Polymerase Chain Reaction. *Molecular Testing in Cancer* (pp. 39–54). New York, NY: Springer New York.

Purwanto, B. H., Kakuda, K., Ando, H., Shoon, J. F., Yamamoto, Y., Watanabe, A., & Yoshida, T. (2002). Nutrient availability and response of sago palm (*Metroxylon sago* Rottb.) to controlled release N fertilizer on coastal lowland peat in the tropics. *Soil Science and Plant Nutrition*, *48*(4), 529–537.

Rampal, L., Jegathesan, M., & Lim, Y. S. (1984). An outbreak of *Bacillus cereus* food poisoning in a school hostel, Klang. *Medical Journal of Malaysia*, *39*(2), 116–122.

Reis, A. L. S., Montanhini, M. T. M., Bittencourt, J. V. M., Destro, M. T., & Bersot, L. S. (2013). Gene detection and toxin production evaluation of haemolysin BL of *Bacillus cereus* isolated from milk and dairy products marketed in Brazil. *Brazilian Journal of Microbiology*, 44(4), 1195–1198.

Reyes, J. E., Bastías, J. M., Gutiérrez, M. R., & Rodríguez, M. O. (2007). Prevalence of *Bacillus cereus* in dried milk products used by Chilean School Feeding Program. *Food Microbiology*, *24*(1), 1–6.

Rosenquist, H., Smidt, L., Andersen, S. R., Jensen, G. B., & Wilcks, A. (2005). Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiology Letters*, *250*, 129–136.

Roy, A., Moktan, B., & Sarkar, P. K. (2007). Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods. *Food Control*, *18*(2), 1555–1564.

Rusul, G., & Yaacob, N. H. (1995). Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. *International Journal of Food Microbiology, 25*(2), 131–139.

Sawei, J., & Sani, N. A. (2016). Prevalence, isolation and characterization of *Bacillus cereus* strains from rice of local cultivators of Sabah, Sarawak, and Peninsular Malaysia. In *2016 UKM FST Postgraduate Colloquium: Proceeding of the Universiti Kebangsaan Malaysia, Faculty of Science and Technology 2016 Postgraduate Colloquium* (Vol. 1784). [030029] American Institute of Physics Inc.

Sandra, A., Afsah-Hejri, L., Tunung, R., Zainazor, T. T. C., Tang, J. Y. H., Ghazali, F. M., & Son, R. (2012). *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat cooked rice in Malaysia. *International Food Research Journal, 19*(3), 829–836.

Sarawak Health Department. (2015). *Sarawak Weekly EPID News Week 41*. Kuching, Sarawak, 2014.

Shi, W., Qingping, W., Jumei, Z., Moutong, C. & Zean, Y. (2015). Prevalence, antibiotic resistance and genetic diversity of *Listeria monocytogenes* isolated from retail ready-to-eat foods in China. *Food Control, 47,* 340-347.

Shin, C., & Collins, J. T. (2015). The Sago Terminology among the Melanau of Sarawak (Malaysia). *Mediterranean Journal of Social Sciences*, *6*(6), 136–144.

Silvester, R., Alexander, D. & Abdullah, M. H. (2015). Prevalence, antibiotic resistance, virulence and plasmid profiles of *Vibrio parahaemolyticus* from a tropical estuary and adjoining traditional prawn farm along the southwest coast of India. *Annal of Microbiology, 65*(4), 2141–2149.

Singhal, R. S., Kennedy, J. F., Gopalakrishnan, S. M., Kaczmarek, A., Knill, C. J., & Akmar, P. F. (2008). Industrial production, processing, and utilization of sago palm-derived products. *Carbohydrate Polymers*, *72*(1), 1–20.

Schlegelova, J., Brychta, J., Klimova, E., Napravnikova, E., & Babak, V. (2003). The prevalence of and resistance to antimicrobial agents of *Bacillus cereus* isolates from foodstuffs. *Veterinarni Medicina*, *48*(11), 331–338.

Sosa, A. D. (2010). Antimicrobial resistance in developing countries. New York: Springer.

Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, *32*(4), 579–606.

Swiecicka, I. (2008). Natural occurrence of *Bacillus thuringiensis* and *Bacillus cereus* in eukaryotic organisms: a case for symbiosis. *Biocontrol Science and Technology*, *18*(3), 221-289.

Svensson, B., Ekelund, K., Ogura, H., & Christiansson, A. (2004). Characterization of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *International Dairy Journal*, *14*(1), 17–27.

Tenover, F. C., Arbeit, R. D., Goering, R. V, & Goering, V. (2012). Epidemiological infections : Review. *Infection Control*, *18*(6), 426–439.

Tortora, G. J., Funke, B. R., & Case, C. L. (2007). *Antimicrobial drugs. Microbiology: An introduction*, 10th Edition. Pearson Education, Singapore.

Toyoda, Y., & for Asian Area Studies, R. D. C. (2008). *Anthropological studies of sago palm in Papua New Guinea*. Ru-Centre for Asian Area Studies. Retrieved from https://books.google.com.my/books?id=gXImAQAAMAAJ

Valero, M., Hernández-Herrero, L. A., Fernández, P. S., & Salmerón, M. C. (2002). Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. *Food Microbiology*, *19*(5), 491–499.

Wang, R. F., Cao, W. W., & Cerniglia, C. E. (1997). A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *Journal of Applied Microbiology*, *83*(6), 727–736.

Waturangi, D. E., Joanito, I., Yogi, Y. & Thomas, S. (2012). Use of REP- and ERIC-PCR to reveal genetic heterogeneity of *Vibrio cholerae* from edible ice in Jakarta, Indonesia. *Gut Pathogens, 4* (2), 1-9.

Weber, D. J., Saviteer, S. M., Rutala, W. A., & Thomann, C. A. (1988). In vitro susceptibility of *Bacillus* spp. to selected antimicrobial agents. *Antimicrobial Agents and Chemotherapy*, *32*(5), 642–645.

Wei, Q.K., Hwang, S. L., & Che, T.R. (2006). Microbiological quality of Ready-to-eat food products in Southern Taiwan. *Journal of Food and Drug Analysis, 14*(1), 68- 73.

Whong, C. M., & Kwaga, J. K. P. (2007). Antibiograms of *Bacillus cereus* isolates from some Nigerian Foods. *Nigerian Food Journal*, *25*(1), 178–183.

World Health Organisation (WHO). (2008). WHO initiative to estimate the global burden for foodborne disease 2014. *Geneva, Switzerland.* Retrieved January 3, 2017, from http://apps.who.int/iris/bitstream/10665/159844/1/9789241507950_eng.pdf

World Health Organisation (WHO). (2014). World health statistics 2014. *Geneva, Switzerland.* Retrieved January 3, 2017, from http://apps.who.int/iris/bitstream/10665/

World Health Organization (WHO). (2016). Antibiotic resistance. *United States of America.* Retrieved December 26, 2016, from http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/

Yim, J. H., Kim, K. Y., Chon, J. W., Kim, D. H., Kim, H. S., Choi, D. S., & Seo, K. H. (2015). Incidence, antibiotic susceptibility, and toxin profiles of *Bacillus cereus sensu lato* isolated from Korean fermented soybean products. *Journal of Food Science*, *80*(6), 1266–1270.

APPENDICES

Appendix 1: Media and Reagent

1.1 Tryptic Soy Broth

Add 30 g to 1 litre of water (purified as required), mix well and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes.

1.2 *Bacillus cereus* Selective Agar Polymyxin pyruvate egg yolk mannitol bromothymol blue (PEMBA) agar

Suspend 20.5 g in 475 ml of distilled water and bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of Polymyxin B Supplement (SR0099) reconstituted as directed, then add 25ml of sterile Egg Yolk Emulsion (SR0047). Mix well and pour into sterile Petri dishes.

1.3 Phosphate Buffered Saline

For 1 liter of 1X PBS, prepare as follows:

i. Start with 800 ml of distilled water.

ii. Add 8 g of NaCl.

iii. Add 0.2 g of KCl.

iv. Add 1.44 g of Na_2HPO_4.

v. Add 0.24 g of KH_2PO_4.

vi. Adjust the pH to 7.4 with HCl.

vii. Add distilled water to a total volume of 1 liter.

Dispense the solution into aliquots and sterilise by autoclaving (20 min, 121°C, liquid cycle). Store at room temperature.

1.4 Slant Agar

Weigh out 50 g of nutrient agar powder. Add it to 1 litre of distilled water. Transfer 5 ml of the solution into universal bottle and sterilise by autoclaving it for 20 minutes at 121 °C. After the agar were sterilised, place the universal bottle on the tilted rack. The agar is allowed to solidify in this position.

1.5 50% Glycerol Stock

Add 500 µL of the overnight culture to 500 µL of 50% glycerol in a 2 ml screw top tube or cryovial and gently mix. Make the 50% glycerol solution by diluting 100% glycerol in distilled water.

Appendix 2: Raw Data for Bacterial Enumeration on *Bacillus cereus* selective agar

Number of bacteria colonies (CFU/ml) of *Bacillus cereus* of the samples from sago processing mill A.

Processing steps	Type of samples	Isolate	Average Number of Bacteria (CFU/ml)
Debarking	Bark swab	SPA 11	5.3×10^2
		SPA 12	3.4×10^3
		SPA 13	-
		SPA 14	2.3×10^3
		SPA 15	-
		SPA 16	5.4×10^2
		SPA 17	3.4×10^4
		SPA 18	7.5×10^3
		SPA19	-

		SPA 20	-
Pulping	Sago pith	SPA 21	-
		SPA 22	4.4×10^2
		SPA 23	-
		SPA 24	-
		SPA 25	-
		SPA 26	-
		SPA 27	-
		SPA 28	5.3×10^4
		SPA 29	2.1×10^2
		SPA 30	-
Waste discharging point	Sago effluent	SPA 61	-
		SPA 62	-
		SPA 63	-
		SPA 64	3.3×10^5
		SPA 65	6.5×10^4
		SPA 66	-
		SPA 67	-
		SPA 68	-
		SPA 69	4.2×10^3
		SPA 70	-

Number of bacteria colonies (CFU/ml) of *Bacillus cereus* of the samples from sago processing mill B.

Processing steps	Type of samples	Isolate	Average Number of Bacteria (CFU/ml)
Debarking	Bark swab	SPB 11	3.7×10^2
		SPB 12	9.4×10^3
		SPB 13	9.4×10^3
		SPB 14	5.3×10^5
		SPB 15	3.2×10^3
		SPB 16	5.4×10^2
		SPB 17	3.4×10^4
		SPB 18	1.2×10^3
		SPB 19	3.2×10^4
		SPB 20	-
Pulping	Sago pith	SPB 21	6.5×10^3
		SPB 22	4.3×10^3
		SPB 23	-
		SPB 24	2.3×10^3
		SPB 25	-
		SPB 26	-
		SPB 27	2.3×10^4
		SPB 28	1.2×10^4
		SPB 29	7.1×10^3
		SPB 30	-
Starch extraction	Starch slurry	SPB 31	2.7×10^4
		SPB 32	-
		SPB 33	-
		SPB 34	-
		SPB 35	3.2×10^2
		SPB 36	-

		SPB 37	3.3×10^3
		SPB 38	-
		SPB 39	8.7×10^3
		SPB 40	-
Drying	Sago milk	SPB 41	-
		SPB 42	-
		SPB 43	-
		SPB 44	-
		SPB 45	-
		SPB 46	-
		SPB 47	2.5×10^3
		SPB 48	2.4×10^2
		SPB 49	-
		SPB 50	-
Waste discharging point	Sago effluent	SPB 61	3.7×10^5
		SPB 62	4.1×10^4
		SPB 63	1.2×10^4
		SPB 64	3.3×10^5
		SPB 65	6.5×10^5
		SPB 66	6.5×10^4
		SPB 67	7.6×10^4
		SPB 68	8.6×10^4
		SPB 69	4.2×10^4
		SPB 70	-

Appendix 3: Raw data for AST

Isolate	DO	C	CRO	F	NOR	KF	K	IPM	E	TOB	S	SXT	P	AMP
SPA 11	15	0	14	14	20	11	22	36	26	19	15	15	0	0
SPA 12	0	0	11	17	20	12	25	32	28	20	17	22	0	0
SPA 14	0	17	0	14	20	12	22	32	30	20	20	15	0	0
SPA 16	18	16	10	20	26	16	10	30	9	22	12	0	0	0
SPA 17	13	9	13	8	23	13	25	28	10	20	14	22	0	0
SPA 18	0	0	0	14	24	14	19	22	18	16	12	13	0	0
SPA 22	9	8	10	14	27	13	9	27	7	24	11	0	0	0
SPA 28	0	15	8	18	26	12	12	30	0	21	10	18	0	0
SPA 29	13	0	16	19	27	12	12	21	0	22	11	19	0	0
SPA 64	9	0	10	15	22	10	22	34	15	20	16	18	0	0
SPA 65	14	20	16	17	21	11	25	25	21	22	21	14	0	0
SPA 69	20	20	13	16	19	11	21	18	17	11	15	12	0	0
SPB 11	0	0	11	13	20	10	21	40	30	19	22	16	0	0
SPB 12	23	20	15	16	22	12	24	29	17	18	21	12	0	0
SPB 13	13	14	15	16	20	14	17	30	29	16	14	10	0	0
SPB 14	17	16	0	17	24	12	15	24	10	15	15	12	0	0
SPB 15	24	21	14	18	22	12	11	20	10	14	13	22	0	0

SPB 16	15	15	15	0	24	0	14	19	25	19	10	16	0	0
SPB 17	25	8	10	12	25	14	18	21	0	15	16	21	0	0
SPB 18	15	9	0	0	20	13	19	23	24	20	12	15	0	0
SPB 19	19	19	13	17	23	15	20	23	9	17	15	17	0	0
SPB 21	10	0	16	7	27	7	11	25	0	23	10	0	0	0
SPB 22	20	20	10	18	21	11	25	34	30	22	24	20	0	0
SPB 24	21	19	8	17	22	10	20	38	15	20	16	18	0	0
SPB 27	12	0	12	12	21	13	22	38	23	17	13	21	0	0
SPB 28	15	0	12	21	29	12	23	38	30	20	17	15	0	0
SPB 29	16	20	15	20	21	11	25	18	17	11	15	12	0	0
SPB 31	17	8	10	18	21	15	18	35	30	15	14	10	0	0
SPB 35	17	9	14	19	22	13	17	40	31	16	14	11	0	0
SPB 37	12	8	16	21	24	13	16	41	21	31	12	21	0	0
SPB 39	12	12	19	18	20	12	27	40	28	20	16	15	0	0
SPB 47	8	17	17	17	21	12	29	36	28	21	23	21	0	0
SPB 48	12	12	17	14	21	12	29	40	34	21	20	15	0	0
SPB 61	18	16	10	20	26	16	10	30	9	22	12	0	0	0
SPB 62	13	19	13	0	23	19	29	30	21	20	14	22	0	0
SPB 63	0	0	0	14	24	14	19	22	18	16	12	13	0	0

SPB 64	9	8	10	14	27	13	9	27	7	24	11	0	0	0
SPB 65	0	15	8	18	26	12	12	30	0	21	10	18	0	0
SPB 66	13	0	16	19	27	12	12	21	0	22	11	19	0	0
SPB 67	9	0	10	19	23	10	22	34	15	20	16	18	0	0
SPB 68	14	20	17	17	21	11	25	25	21	22	21	14	0	0
SPB 69	20	20	13	19	19	11	14	19	25	19	15	12	0	0