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Detection, genetic diversity and antibiotic resistance profiles of *Bacillus cereus* isolated from sago processing plants in Malaysia

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ABSTRACT

Aims: *Bacillus cereus* is a Gram-positive, rod-shaped and spore-forming bacterium. It is a ubiquitous bacterium which is widely distributed in several environments such as soil and plants and is commonly isolated from food and its processing environment. This study was aimed to determine the genetic diversity and antibiotic resistance of *B. cereus* isolated from sago processing in Sarawak.
Methodology and results: Out of 120 samples, 42 *B. cereus* isolates were detected with the presence of *hly* gene of *B. cereus* by using specific polymerase chain reaction (PCR). Twenty *B. cereus* isolates were randomly selected and further characterized by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA digested with *Not*I to examine the genetic diversity. The result of the PFGE analysis confirmed that the *B. cereus* strains in sago processing were genetically diverse. Based on the dendrogram generated, *B. cereus* strains were grouped into two major clusters and these clusters were grouped together based on sources of isolation. The investigation on the antibiotic resistance of *B. cereus* strains revealed that the *B. cereus* strains were uniformly highly resistant to penicillin and ampicillin and highly susceptible to imipenem and norfloxacin.
Conclusion, significance and impact of study: The results of this study suggest that the *B. cereus* isolated from sago processing derived from a mixture of sensitive and resistant strains with diverse genetic contents.

Keywords: B. cereus, sago processing, genetic diversity, PFGE, antibiotic resistance

INTRODUCTION

Sago processing industry is a high potential industry in Sarawak and are grown commercially in Malaysia, Indonesia, Philippines and New Guinea (Bujang, 2008). Sago is an important food for millions of people (Adeni *et al.*, 2010). In Malaysia, approximately 90% of sago planting area can be found in Sarawak and 75% are in Mukah, Sarawak (Bujang, 2010). Sago industries in Sarawak have great contribution to Sarawak economy whereby the export of sago product from Sarawak procuring income approximately RM 81 million per year (Department of Agriculture Sarawak, 2013). However, in food processing industries, some of the preparation, processing and storage procedures were exposed to the risk of contamination of bacterial contaminants. *B. cereus* is one of the known causes of foodborne illness. *B. cereus* is a Gram-positive, spore-forming and motile rod-shaped bacterium that can cause gastrointestinal diseases such as food poisoning (Kotiranta *et al.*, 2000; Sandra *et al.*, 2012). Hence, it is important to monitor their presence in foodstuffs by using rapid and reliable molecular method (Merzougui *et al.*, 2013). To the best of our knowledge, this is the first study on the genetic diversity of *B. cereus* isolated from the processing steps of sago in Sarawak by PFGE method. However, the study of genetic diversity of *B. cereus* by PFGE had been reported in other food products/processing plants such as in milk, spices, rice salad, yogurt and cheese (Merzougui *et al.,* 2014), rice and ready-to-eat cereal (Park *et al.,* 2009) and chilled zucchini puree processing plant (Guinebretiere *et al.,* 2003) whereby all these studies revealed the genetic differences among the *B. cereus* from the samples isolated. Hence, present study was aimed to investigate the diversities of *B. cereus* in sago processing by PFGE method. In addition, the antibiotic resistance of *B. cereus* strain was also examined.

MATERIALS AND METHODS

Sample collection and preparation

A total of 120 samples comprise of bark swab (n=20), sago pith (n=20), sago effluent (n=20), starch slurry (n=20), sago milk (n=20) sago flour (n=20) were collected from sago processing mill in Sarawak. Approximately 50

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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. Samples were transported to the lab by using ice box containing ice within 24 h. Samples were analyzed using the standard procedure for the detection of *B. cereus* according to Bacteriological Analytical Manual by Food and Drug Administration USA (Sandra *et al.,* 2012). Ten grams of each sample was weighed in sterile condition and placed in a stomacher bag added with 90 mL of Tryptic Soy Broth (TSB) and homogenized accordingly. Then, the samples were incubated in 37 °C for 24 hours. After an overnight incubation, a loopful of culture was streaked on *B. cereus* selective agar (Himedia) and were incubated for 24 h at 37 °C. Plates that contain presumptive positive with the presence of peacock blue colonies on peacock blue medium were subjected to specific polymerase chain reaction (PCR) by targeting *hly* gene (5'-CTGTAGCGAATCGTACGTATC-3' and 3'-TACTGCTCCAGCCACATTAC-5') of *B. cereus* that encode the toxin producing gene in *B. cereus.*

Pulsed-field gel electrophoresis (PFGE) genotyping

Out of 120 samples, a total of 42 *B. cereus* strain collected from debarking (n=15), pulping (n=9), starch extraction (n=4), drying (n=2) and discharging (n=12) were detected with *hly* gene of *B. cereus.* The PFGE protocol was adapted and optimized from Merzougui *et al.* (2014). Twenty *B. cereus* pure isolates were randomly chosen and *B. cereus* reference strain ATCC 14579 were inoculated in Luria Bertani (LB) broth for 24 hours at 37 °C. One milliliter of Tris-EDTA (TE) buffer (pH 8, 0.01 M) 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. Ten microlite of lysozyme (20 mg/mL) were added to the cell suspension and was incubated for 15 m 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. One point five milliliter of cell lysis buffer were dispensed into 1.5 mL microcentrifuge tube and labelled accordingly. The plugs were incubated into 54 °C water bath with constant 150 ×g agitation 2 h. DNA was digested with the enzyme *Not*I (20 U) following migration performed with the CHEF DRII BioRad system (BioRad) in a 1.5% agarose gel in 0.5× TBE buffer at 14 °C with a linear ramping time of 2.4-60.8 s over a period of 20 h, and a gradient of 200 V/cm. After migration, the gels were stained with ethidium bromide and photographed under UV transilluminator. PFGE patterns obtained 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).

Antibiotic susceptibility test

All 42 *B. cereus* strain were subjected to antibiotic susceptibility test. 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 (10µg), 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 Schlegelova *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). The *B. cereus* strains were rated as susceptible, intermediate and resistant.

RESULTS

PCR assay

Out of 120 samples tested, 42 samples were positive with *B. cereus.* The prevalence of *B. cereus* associated with sago processing was summarized in Table 1.

Table 1: Prevalence of *B. cereus* strains isolated from each step of sago processing.

Processing step	Number of samples collected	Positive with *B. cereus*	Prevalence (%)
Debarking	20	15	75
Pulping	20	9	45
Starch extraction	20	4	4
Drying	20	2	2
Packaging	20	0	0
Discharge	20	12	60
TOTAL	120	42	35

PFGE analysis

The dendrogram shown in Figure 1 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.

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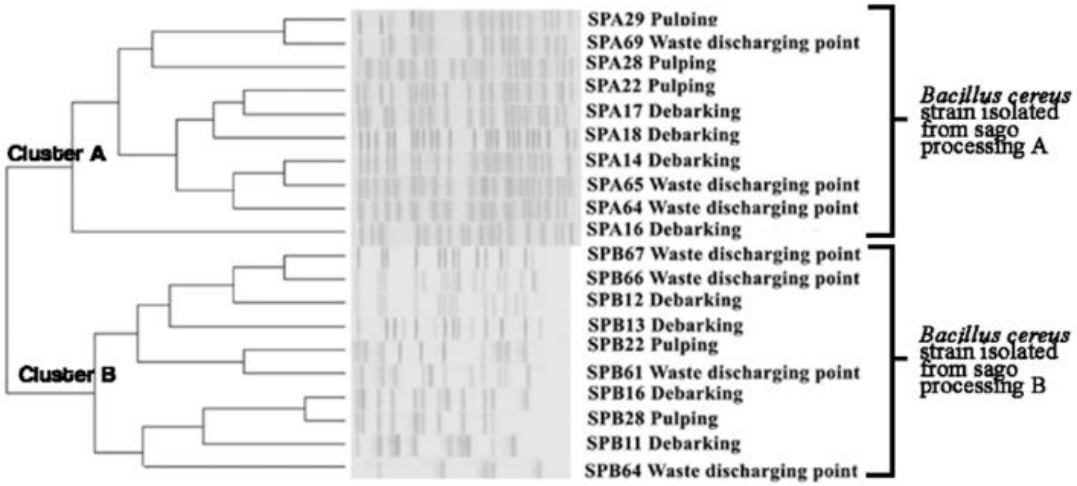

Figure 1: Dendrogram of PFGE profile from *Not*I digestion pattern of *B. cereus* strains isolated from each step of sago processing. *B. cereus* strains were grouped based on source of samples collected.

Antibiotic resistance

The summary of the percentage of bacterial resistance based on antibiotics are shown in Table 2. All isolates were highly resistance to ß-lactam antibiotics penicillin (100%), and ampicillin (100%). On the other hand, all isolates show the highest level of susceptibility towards imipenem (100%), norfloxazin (100%), tobramycin (96.67%).

Table 2: Antibiotic resistance of *B. cereus* strains isolated from each step of sago processing.

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	42	42	100.0
Penicillin	42	42	100.0

DISCUSSION

The isolation of *B. cereus* of from sago processing is consistent with the ecology of the *Bacillus* genus and usually associated with raw foods of plant origin. *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 wide range of sites such as milk, dairy, ready-to-eat cereals and rice (Kotiranta *et al*., 2000; Greenhill *et al.*, 2010; Lesley *et al*., 2013). However, *B. cereus* usually was linked with farinaceous and starchy products such as rice and sago starch (Greenhill *et al.,* 2010). *B. cereus* causes problems to the foodstuff industry both by deteriorating the products (Eneroth *et al.,* 2001; Sandra *et al.,* 2012; Lesley *et al.,* 2013), and by endangering people's health upon consuming contaminated foods (Ghelardi *et al.,* 2002).

The results of this study indicate that the *B. cereus* strains were grouped based on the sources of isolation. This is comparable to a study by Merzougui *et al.* (2014). Their study revealed that there was visible correlation between the PFGE DNA fingerprint profiles and sources of isolates. Similar strains with one or two band differences showed that the *B. cereus* strain were possibly clonally related. In addition, genomic variability of *B. cereus* strains from some stage showed more than one PFGE pattern produced. This is in line with previous study by Guinebretiere and Nguyen (2003) and Merzougui *et al.* (2013). They suggested that it might due to multiple sources of contamination occurred at single site of isolation.

The summary of the percentage of bacterial resistance based on antibiotics are shown in Table 1. All isolates were highly resistance to ß-lactam antibiotics penicillin (100%), and ampicillin (100%). High penicillin and ampicillin resistant were also observed in *B. cereus* in fermented soybean (Kim *et al.*, 2015). Andrews and Wise (2002) and Park *et al.* (2009) also reported that *B. cereus* were highly resistant to penicillin and ampicillin. High resistance towards penicillin and ampicillin were not surprising as there were among the first antibiotics introduced since the discovery of penicillin (Tortora *et al.,* 2007). This also could be explained by the fact that *B.*

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cereus is genetically resistant to all ß-lactam except carbapenems (Ikeda *et al.*, 2015) and have been caused by enzymatic modification of agents (Schlegelova *et al*., 2003). High resistance towards penicillin, ampicillin and other ß-lactam were influenced by the production of β-lactamases enzyme in *B. cereus* (Kotiranta *et al*., 2000; Chen *et al*., 2004). The enzyme deactivates 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 (Jalalpour and Ebadi, 2012). There are three forms 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 *et al*. (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 (Jalalpour and Ebadi, 2012). On the other hand, all isolates show the highest level of susceptibility towards imipenem (100%), norfloxazin (100%), tobramycin (96.67%). The results were in close agreement to a study by Andrews and Wise (2002), Park *et al*. (2009) and Kim *et al.* (2015). It is not surprising since imipenem could be prescribed by doctors as a treatment for bacterial infection (Letchumanan *et al.,* 2015).

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 (Rosenquist *et al.,* 2005). This is the first study on the genetic diversity of *B. cereus* isolated from the processing steps of sago in Sarawak by PFGE method. The antibiotic resistance patterns of *B. cereus* isolates from Korean fermented soybean products are therefore consistent with earlier findings. 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 (Alum *et al.,* 2016).

CONCLUSION

In this research, molecular typing by using PFGE was 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. This study suggests 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, good hygiene practice (GHP) and good manufacturing practice (GMP) must be adopted in sago processing to avoid unwanted illness due to consumption of contaminated sago and sago products. Antibiotic resistance profile of *B. cereus* was also determined in this study. The percentage of the susceptibilities and resistance of *B. cereus* towards antibiotics 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.

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REFERENCES

Adeni, D., S, Aziz, S., Bujang, K. and Hassan, M. A. (2010). Bioconversion of sago residue into value added products. *African Journal of Biotechnology* ***9*****(14), 2016-2021.**

Alum, E. A., Urom, S. M. O. C. and Ben, C. M. A. (2016). Microbiological contamination of food: The mechanisms, impacts and prevention. *International Journal of Scientific and Technology Research* **5(3), 65-78.**

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

Bujang, K. (2008). Potentials of bioenergy from the sago industries in Malaysia. *In:* Biotechnology: Fundamentals in Biotechnology. Doelle, H. W., Rokem, S. and Berovic, M. (eds.). Vol XIV. Encyclopedia of Life Support Systems. Oxford, UK.

Bujang, K. (2010). Sago: A food and fuel alternative. In: Rahman, M. A., Yee, L. T., Bojo, O. Puad, A. S. A, Ho, W. S., Azad, A. J. A. G., and Rakawi, F. F. (eds.). Proceedings of BioBorneo 2014: Sustaining the Bioeconomy Community. i-FoRST, Kota Samarahan, Malaysia. **pp. 141-145.** [Retrieved on 2 January 2017]

Chen, Y., Tenover, F. C., and Koehler, T. M. (2004). Beta-lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. *Antimicrobial Agents and Chemotherapy* **48(12), 4873-4877.**

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

Department of Agriculture Sarawak. (2013). Sarawak Agriculture Statistics 2013. Malaysia. Available from http://www.doa.sarawak.gov.my/modules/web/pages.php?mod=webpageandsub=pageandid=712 [Retrieved 2 January 2017].

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

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

Greenhill, A. R., Shipton, W. A., Blaney, B. J., Amoa, B., Kopel, E., Pelowa, D. and 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. and Nguyene, C. (2002). Enterotoxigenic profiles and food poisoning and foodbome *B. cereus* strains. *Journal of Clinical Microbiology* **40(2), 3053-3056.**

Guinebretiere, M. H. and Nguyen-The, C. (2003). Sources of *B. cereus* contamination in a pasteurized zucchini puree processing line, differentiated by two PCR-based methods. *FEMS Microbiology Ecology* **43(2), 207-215.**

Ikeda, M., Yagihara, Y., Tatsuno, K., Okazaki, M., Okugawa, S. and Moriya, K. (2015). Clinical characteristics and antimicrobial susceptibility of *B. cereus* blood stream infections. *Annals of Clinical Microbiology and Antimicrobials* **14, 43.**

Jalalpour, S. and 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.**

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 *B. cereus* Isolated from Korean fermented soybean products. *Journal of Food Science* **80(1), 123-128.**

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

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

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

Merzougui, S., Lkhider, M., Grosset, N., Gautier, M. and Cohen, N. (2013). Differentiation by molecular typing of *B. cereus* isolates from food in Morocco: PFGE-Eric PCR. *Food and Public Health* **3(4), 223–227.**

Merzougui, S., Lkhider, M., Grosset, N., Gautier, M. and Cohen, N. (2014). Prevalence, PFGE typing, and antibiotic resistance of *B. cereus* group isolated from food in Morocco. *Foodborne Pathogen and Disease* **11(2), 145-149.**

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

Rosenquist, H., Smidt, L., Andersen, S. R., Jensen, G. B. and Wilcks, A. (2005). Occurrence and significance of *B. cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiology Letters* **250(1), 129-136.**

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

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

Tortora, G. J., Funke, B. R. and Case, C. L. (2007). Antimicribial drugs. *In:* Microbiology: An introduction (10th Edn). Pearson Education, Singapore.

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