ผลของความเข้มข้นของตัวยับยั้งและปริมาณเซลล์เริ่มต้นต่อการเพิ่มจำนวนเชื้อบาซิลลัส

EFFECT OF INHIBITORS AND INITIAL CELL CONCENTRATIONS ON THE ENRICHMENT *BACILLUS CEREUS*

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บทคัดย่อ

โดยปกติแล้วกระบวนการคัดเลือกเชื้อจุลินทรีย์บาซิลลัสซีเรียสจะใช้เพียงโพลีมัยซินบีซัลเฟต ความเข้มข้น 10 มิลลิกรัมต่อลิตรเท่านั้น ที่ความเข้มข้นดังกล่าวเชื้อบาซิลลัสจะเจริญได้ตามปกติ แต่เชื้อแข่งขันกลุ่มแกรมบวกอื่น ๆ ก็สามารถเจริญได้เช่นกัน ส่งผลให้เกิดความลำบากในการวิเคราะห์ผล โดยเฉพาะอย่างยิ่งกับตัวอย่างในอุตสาหกรรมที่ต้องการความถูกต้องและรวดเร็ว

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของจำนวนเชื้อจุลินทรีย์เริ่มต้นและความเข้มข้นของสารยับยั้งชนิดต่าง ๆ ที่มีผลต่อการเจริญของเชื้อจุลินทรีย์บาซิลลัสในอาหารเลี้ยงเชื้อที่ทำการคัดเลือกชนิดเหลวสารยับยั้ง 3 ชนิด ที่ใช้ในการทดลอง ได้แก่ โพลีมัยซินบีซัลเฟต อะม็อกซีซิลลิน และเซฟไตรอะโซน ที่ระดับความเข้มข้นเท่ากับที่ใช้ตามมาตรฐาน (10 มิลลิกรัมต่อลิตร) ครึ่งหนึ่งของมาตรฐาน (5 มิลลิกรัมต่อลิตร) และสูงกว่ามาตรฐานหนึ่งเท่า (20 มิลลิกรัมต่อลิตร) ความเข้มข้นของเชื้อบาซิลลัสที่ศึกษา ได้แก่ 10^1 10^3 และ 10^5 โคโลนีต่อมิลลิลิตร โดยทำการศึกษาในอาหารเหลวชนิด Tryptic Soy Broth (TSB) และทำการนับจำนวนบนอาหารเลี้ยงเชื้อชนิด Tryptic Soy Agar (TSA) หลังจากบ่มที่อุณหภูมิ 30 องศาเซลเซียส นาน 24 ชั่วโมง

ผลวิจัยพบว่า เซฟไตรอะโซนที่ความเข้มข้น 20 มิลลิกรัมต่อลิตร เป็นสารยับยั้งที่ให้ผลดีที่สุด ซึ่งไม่ส่งผลกระทบต่อการเจริญของบาซิลลัส สำหรับอะม็อกซีซิลลินออกฤทธิ์ในการยับยั้งบาซิลลัสอย่างรุนแรงที่ความเข้มข้นเชื้อต่ำในทุก ๆ ความเข้มข้นของสารยับยั้ง

โดยทั่วไปความเข้มข้นของสารยับยั้งที่มากจะไปเพิ่มความเป็นพิษของอาหารจำเพาะและมีผลกับความเข้มข้นของเซลล์เริ่มต้นที่น้อยและมีผลโดยตรงกับการฟื้นตัวเพิ่มจำนวนของ *B. cereus* ที่ปริมาณความเข้มข้นของเซลล์เริ่มต้นน้อยทำให้เกิดการเจริญที่มีลักษณะ Lag phase ที่ยาวนานและบางครั้งให้การแปรผลที่ผิดพลาด (False negative) เนื่องจากผลของตัวยับยั้งที่มีความแรงมาก ดังนั้นการเลือกใช้สารคัดเลือกที่เหมาะสมจึงมีความสำคัญ

คำสำคัญ: บาซิลลัสซีเรียส โพลีมัยซินบีซัลเฟต อะม็อกซีซิลลิน เซฟไตรอะโซน ความเข้มข้นของสารยับยั้ง ความเข้มข้นเริ่มต้นของเซลล์

Abstract

Conventionally, the selectivity of *Bacillus cereus* in the enrichment step is only limited to the addition of 10 mg/L Polymyxin B sulfate. At this concentration, the growth of *B. cereus* is compromised and its selectiveness against other competing Gram-positive bacteria for industrial food samples is disputable. This research was aimed to study the effects of initial cell concentration and concentration of selective inhibitors on the recovery of *B. cereus* in selective enrichment step.

Method: The inhibitory effect of three conventional and alternative inhibitors (i.e., Polymyxin B sulfate, amoxicillin, and ceftriaxone) was evaluated at different initial cell densities (10^1, 10^3, and 10^5 CFU/mL) to determine the optimal *B. cereus* inhibitors and their suitable dosage. These selective inhibitors were added to Tryptic Soy Broth (TSB) and the recovery of *B. cereus* was evaluated using viable cell count on Tryptic Soy Agar (TSA). The concentrations of these selected inhibitors were formulated by doubling the suggested strength (recommended by most standard enrichment protocols) and reducing the standard concentration by half. Hence, the treatment of inhibitors was varied at 5, 10, and 20 mg/L. The cell recovery was performed at 30°C for 24 h.

Result: In this research, ceftriaxone showed good preference towards the growth of *B. cereus* in TSB with ceftriaxone at 20 mg/L and significant toxicity against *E. coli* and *S. aureus*. At low initial cell concentrations, no viable *B. cereus* cell was detected in all amoxicillin treatments.

Conclusion: Generally, higher concentrations of the selective inhibitors increased the toxicity of the selective broth and the initial cell concentration resulted in a subtle consequence and directly affected the recovery of *B. cereus*. The lower initial cell concentrations produced longer lag phase and sometimes produced false negative results due to the inhibitory effect of the strong inhibitor. The selectivity for *B. cereus* has to improve.

Keywords: *Bacillus cereus*, Polymyxin B sulfate, Amoxicillin ceftriaxone, Inhibitor concentration, Initial concentration

Introduction

There are many food qualities, which are the main concerns for food industry; first is sensorial qualities. Second one is physico-chemical properties and finally microbiological qualities. Microbiological

qualities become main food manufacturer concern due to their severity, which caused a lot of outbreaks in recent time. Food borne illnesses are caused by various foodborne pathogenic microorganisms depend on their origin and type of foods. *B. cereus* has emerged as a critical food-borne pathogen frequently responsible for an increasing number of product recalls in the industrial world [1]. When contaminated in human's gastrointestinal tract, *B. cereus* can produce two types of toxins, diarrheal syndrome toxin and emetic syndrome toxin [2]. A study of common supermarket food items in Czech Republic showed the contamination with *B. cereus* strains was 31% of all dairy products and 28% of meat products samples [3]. In 2009, a mass recall of all canned Slim-Fast® ready-to-drink products in the US was exercised to prevent the possibility of *B. cereus* outbreak [4].

Currently, the recommended method by universal food regulatory agencies (i.e., Food and Drug Administration, BAM, ISO, and AOAC) for *B. cereus* identification relies on Mannitol Egg Yolk Polymyxin (MYP) media followed by biochemical characterization [5]. Based on our preliminary experiment, some problems may occur during the detection of *B. cereus* using this selective agar medium. The isolation was prone to the growth of other background flora such as *Staphylococcus aureus* or other non-pathogenic *Bacillus* species causing misinterpretation of phospholipase C activities on this egg yolk medium as also observed by Tallent and others [5]. Perhaps, only 10 mg/mL Polymyxin B sulfate as a selective agent was not enough to discourage other bacterial competitors. Polymyxin B sulfate was first applied as a selective agent for *B. cereus* isolation in the study of Donovan [6] to increase the selectivity in milk samples. Since that day, Polymyxin B is always used as a selective agent for *B. cereus* determination. However, Polymyxin B is works against only Gram negative bacteria so other Gram positive still able to grow in these media cause misinterpretation of those results. Therefore, other alternative selective agents are required to improve the media selectivity. Ceftriaxone is a third-generation cephalosporin antibiotic. Like other third-generation cephalosporins, it has broad spectrum activity toward Gram-positive and Gram-negative bacteria. Even some antibiotic resistance on ceftriaxone in *Salmonella* spp. was recently reported [7], though combining ceftriaxone with other antibacterial agent could eliminate this thread. *Bacillus cereus* and *Clostridium* spp. showed very resistant to ceftriaxone in milk samples [8]. Amoxicillin is a β-lactam antibiotic which has an effect on the alteration of bacterial cell wall by forming cross-linkage between linear peptidoglycan of both Gram positive and Gram negative. The bacteria in the spectrum of amoxicillin are *Bacillus subtilis* and *Staphylococus* spp. [9]. *B. cereus* showed moderate sensitivity on amoxicillin [10]. There are other antibiotics successfully applied to isolate *B. cereus* in agar culture. Supplementing MYP agar with trimethoprim was reported to improve selectivity of *B. cereus* in food samples with high background of micro flora [11]. Also novobiocin was reported to effectively inhibit the *Bacillus subtilis* growth and spore germination due to the disruption of RNA synthesis [12]. Moreover several studies of antibiotic resistant in *B. cereus* were reported such as cephalosporin group [13] and amoxicillin [10].

Objectives

The main goal of this research was to study the effect of types and concentrations of selective agents on *Bacillus cereus* growth and against its competitive microorganisms upon different initial cell

concentrations. The information obtained from this research could provide a guideline for decreasing the enrichment step and cost when comparing to the routine protocols. Moreover, the techniques in this research was to improve the effectiveness of enrichment media in fostering *Bacillus cereus* growth and selectivity.

Methods

Bacterial strains and culture preparation; *B. cereus* strain ATCC 118878 and other competing bacteria (*E. coli* DMST 4609 and *S. aureus* TISTR 808) were obtained from either the Department of Medical Sciences Thailand (DMST, Bangkok, Thailand) and Thailand Institute of Scientific and Technological Research (TISTR, Bangkok, Thailand). All pure cultures were used for the experiments and maintained on Tryptic Soy Agar (TSA, Lab M, UK) at 4°C. One loopful of each strain was transferred into 100 mL of Tryptic Soy Broth (TSB, Lab M, UK) and incubated at 30°C (200 rpm, 24 h). Cell suspensions were serially diluted by 10 fold to meet the final cell concentrations (at approximately 10^1, 10^3, and 10^5 CFU/mL).

Study of alternative selective agents; Three antibiotics including Polymyxin B sulfate, amoxicillin, and ceftriaxone were prepared at different concentrations (5, 10, and 20 mg/L) in TSB medium. The concentration of Polymyxin B sulfate at 10 mg/L is considered as a common level from the recommendation of many food safety standards [14]. Different initial cell suspensions of *B. cereus* were inoculated into each modified TSB well in 2 mL cultivation volume. Viable cell density was counted with modified method [15] at 0, 4, 8, 12, 16, and 24 h on TSA with proper dilution with tryptone salt diluents (1 g/L tryptone, 8.5 g/L NaCl) before plating and incubated at 30°C in hot air incubator. Briefly, the cultivation volume for plating was reduced from 100 mL to 10 µL and plating on agar which loaded in 96 well plates. All serial dilutions were also done in 96 well plates with the ratio of 20 : 180 µL. Three replications were conducted in this experiment. The cell densities at different incubation time were calculated the percent inhibition as follows the equation 1.

Study of inhibition effects; Three antibiotics were prepared at different concentrations (5, 10, and 20 mg/L) in TSB medium. The initial cell suspensions at 10^5 CFU/mL (*B. cereus, E. coli, and S. aureus)* were inoculated into each modified TSB well in 2 mL cultivation volume. Viable cell density was counted with modified method at 24 h on TSA with proper dilution with tryptone salt diluents (1 g/L tryptone, 8.5 g/L NaCl) before plating and incubated at 30°C in hot air incubator.

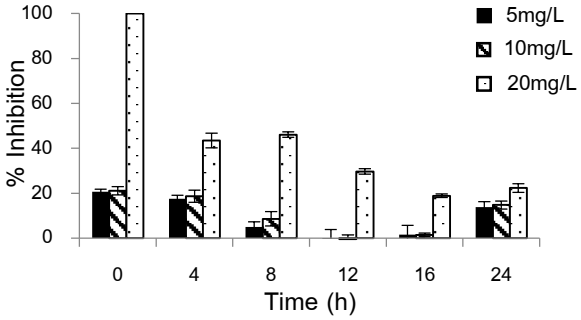
Percent inhibition = (viable cells in TSB w/o inhibitors (CFU/mL) - viable cells in TSB with inhibitors (CFU/mL))/viable cells in TSB w/o inhibitors (CFU/mL) (Eq. 1)

Results

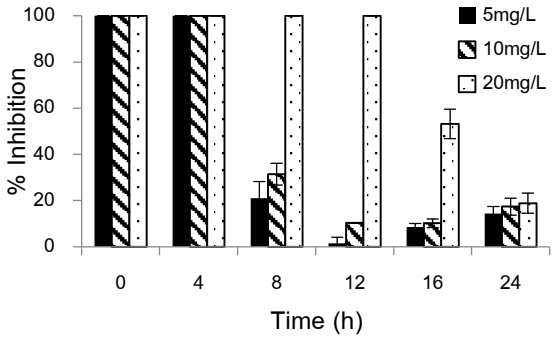
The effect of different concentration of selective agents

Polymysin B sulphate

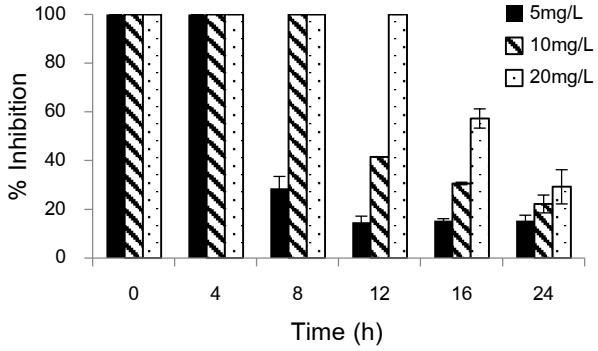
When Polymyxin B sulfate was supplemented in the TSB, it produced different degrees of inhibitory effect on *B. cereus* growth characteristics described by percent inhibition (Eq. 1) depending on the initial cell and concentrations.



a) Innoculum culture at 10^5 CFU/mL



b) Innoculum culture at 10^3 CFU/mL



c**)** Innoculum culture at 10^1 CFU/mL

Figure 1. Percent of *B. cereus* inhibition in TSB supplemented with various concentrations of Polymyxin B sulfate at different initial concentrations a) 10^5 CFU/mL, b) 10^3 CFU/mL, and c) 10^1 CFU/mL.

At higher initial cell loading (10^5 CFU/mL) treatments, *B. cereus* cells were less inhibited by Polymyxin B, especially at lower concentrations of Polymyxin B (Fig. 1c). Using 5-10 mg/L of Polymyxin B sulfate, the percent inhibition of the 10^5 CFU/mL treatment was kept relatively less than 20% (Fig. 1a). For low initial cell loading, the effect of Polymyxin B sulfate was more pronounced even at low dosages of this inhibitor (Fig. 1b-c). The 20 mg/L Polymyxin B treatment was generally too toxic for *B. cereus* recovery. However, the inhibitory effect gradually subsided toward the end of the incubation period.

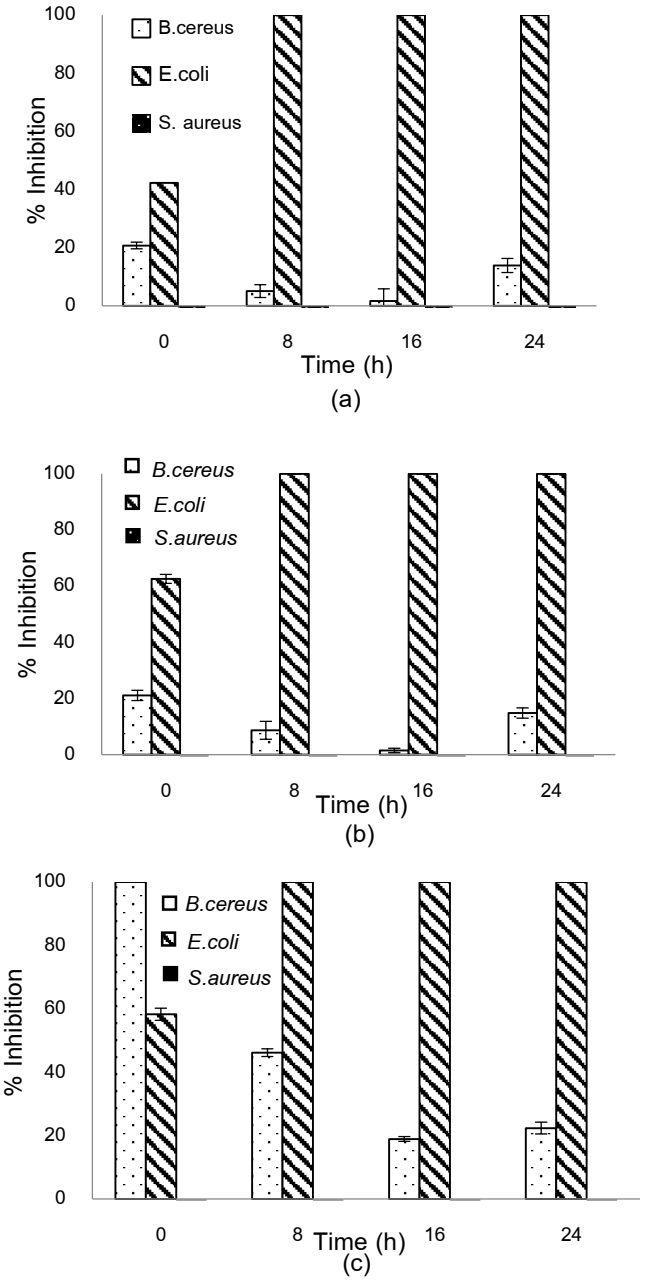
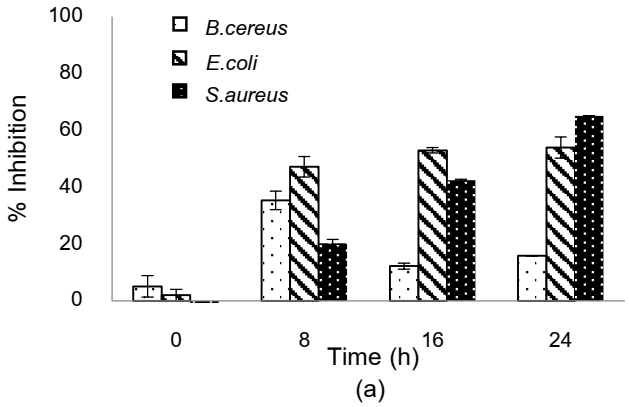


Figure 2. Percent of microorganisms (*B. cereus*, *E. coli*, and *S. aureus*) inhibition in TSB supplemented with Polymyxin B sulfate at different concentration a) 5 mg/L b) 10 mg/L c) 20 mg/L.

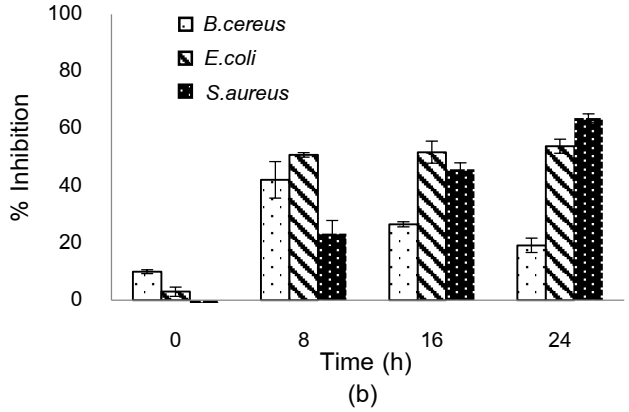
The effect of Polymyxin B sulfate against the growth of *B. cereus, E. coli,* and *S. aureus* were shown in Figure 2. At 5 mg/ L of Polymyxin B treatment, *E. coli* showed very high sensitivity to Polymyxin B and its growth was totally suppressed at the first 8 h of cultivation period. On the other hand, antagonistic result was observed for *S. aureus,* which belong to Gram-positive bacterial strains, showed very endurable to the toxicity of Polymyxin B sulfate at this concentration. The similar results were remarked when increase the concentration of Polymyxin B from 5 to 10 mg/L (Fig. 2b). At 20 mg/L (Fig. 2c), Polymyxin B produced some negative effect on *B. cereus* growth in the initial phase of cultivation. Toward the end of this experiment, the effect of Polymyxin B was even more to *E. coli* followed by *B. cereus* but left unaffected to *S. aureus.*

Amoxicillin

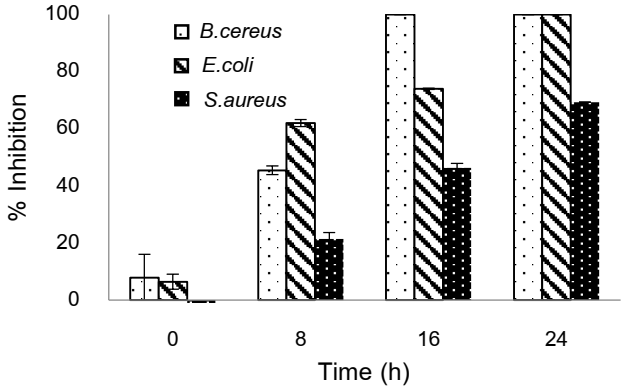
Even though, amoxicillin comparatively produced a good selection to *B. cereus* but it produced only moderate effect to screen out *E. coli* and *S. aureus* at 5 (Fig. 3a) and 10 mg/L (Fig. 3b).



(a)



(b)



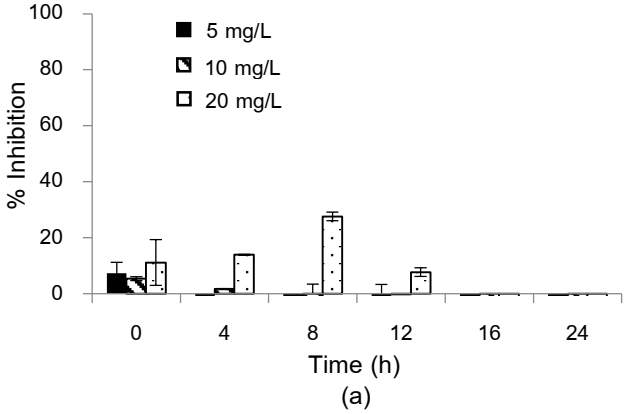
(c)

Figure 3. Percent of microorganisms (*B. cereus*, *E. coli,* and *S. aureus*) inhibition in TSB supplemented with amoxicillin at different concentration a) 5 mg/L b) 10 mg/L c) 20 mg/L.

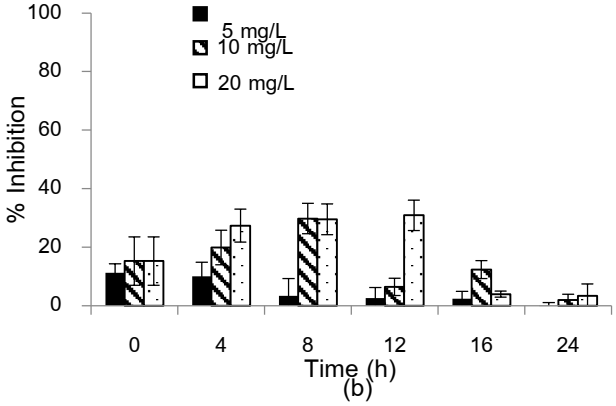
When the concentration of amoxicillin was increased to 20 mg/L (Fig. 3c), the reverse effect was observed toward *B. cereus* growth. At 20 mg/L, the growth of *B. cereus* was more suppressed than other microbes.

Ceftriaxone

The inhibitory effect of ceftriaxone toward *B. cereus* growth was reported in Figure 4. At high initial cell concentration, 10^5 and 10^3 CFU/mL (Fig. 4a and 4b), the inhibitory effect was rarely marked compare with another cell level; 10^1 CFU/ mL (Fig. 4c) . The concentration of ceftriaxone revealed at high concentration in the first 12 h and declined when the growth approach to stationary phase.



(a)



(b)

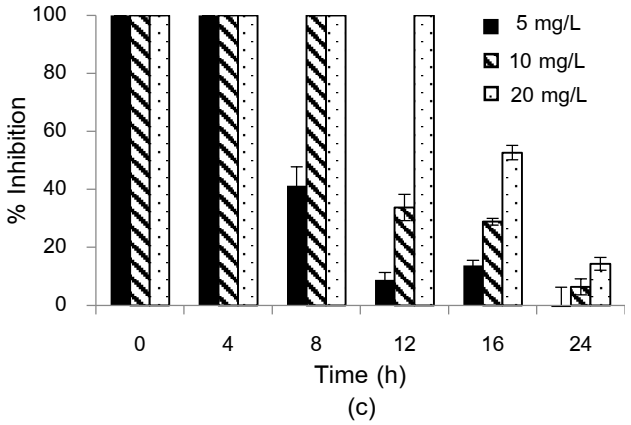
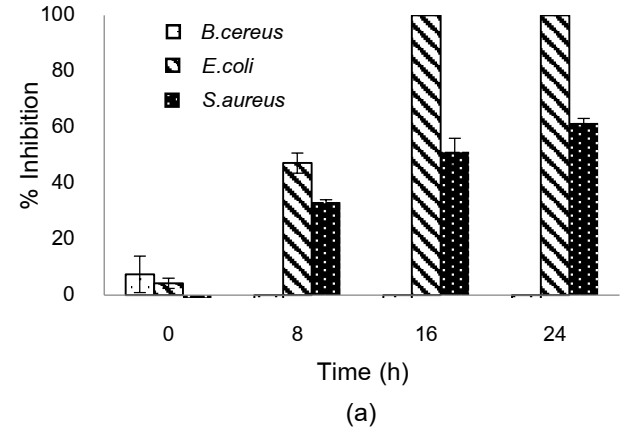
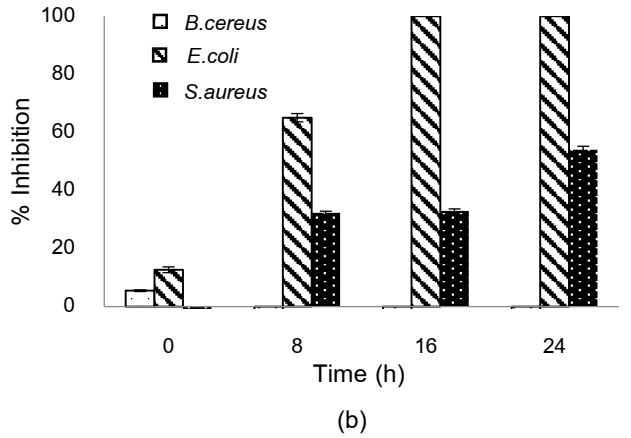


Figure 4: Percent of *B. cereus* inhibition in TSB supplemented with various concentrations of ceftriaxone at different initial concentrations a) 10^5 CFU/mL, b) 10^3 CFU/mL, and c) 10^1 CFU/mL).

For the selectivity of *B. cereus* against other emulous microorganisms, ceftriaxone seem to be the best choice among the 3 other inhibitors which used in this experiment.





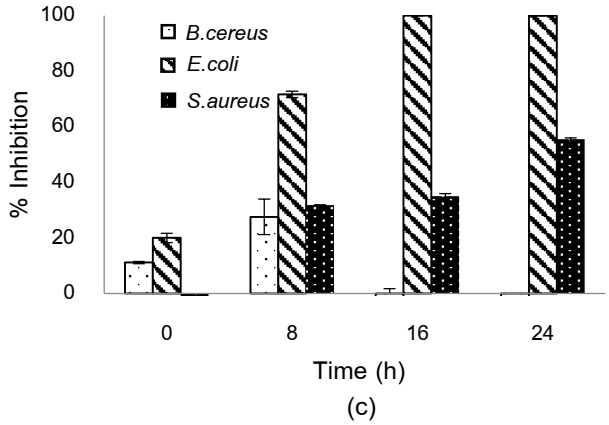


Figure 5: Percent of microorganisms inhibition (*B. cereus*, *E. coli*, and *S. aureus*) in TSB supplemented with ceftriaxone at different concentration a) 5 mg/L b) 10 mg/L c) 20 mg/L.

Ceftriaxone produced strongest negative effect on the *E. coli* and some effect on *S. aureus* at 5 mg/L (Fig. 5a). The same trend were also observed when increase the concentration of ceftriaxone to 10 and 20 mg/L (Fig. 5b and 5c). However, the use of 20 mg/L (Fig. 5c) also produced some inhibitory effect on *B. cereus* growth during the early log phase.

Conclusions and Discussion

The use of Polymyxin B sulfate was well-documented to inhibit on a wide array of bacteria other than the *Bacillaceae* in food samples [16]. The typical concentration of 10 mg/L has been applied to many selective enrichment media of *B. cereus*, including ISO 21871, BAM, AOAC, and AFNOR. The use of Polymyxin B sulfate (at 10 mg/L) was confirmed to be fairly effective for *B. cereus* isolation at high initial cell loading. However, this level of Polymyxin B sulfate was almost too toxic for lower initial cell loading (i.e., 10^1 CFU/mL or less). If the level of *B. cereus* presence was too low, the lower Polymyxin B sulfate concentration at 5 mg/L may prove more effective to resuscitate *B. cereus* in the selective enrichment step. However, further study has to be conducted to optimize the potent concentration to discourage other competing Gram-negative microorganisms. Also the lower concentration of Polymyxin B sulfate may compromise the isolation of *B. cereus* over other Gram-positive bacteria since it has been well-documented that Polymyxin B sulfate had no activity against Gram-positive bacteria and anaerobes [17].

The use of 20 mg/L of amoxicillin leads to cell death after 12 h of incubation in Figure 3. Amoxicillin seems to be a strong inhibitor due to its negative activity toward *B. cereus* on 10^3 and 10^1 CFU/mL but it also inhibits the growth of on *E. coli* and *S. aureus* as well. Even though there were some reports on amoxicillin-resistant in *B. cereus*, but the number of resistant was reported to be 60% of all tested strains [18]. Therefore, amoxicillin does not provide a good selectivity for the *B. cereus* in some strains.

The ability of B. cereus to withstand the toxicity of ceftriaxone was also reported by Ko and other [19] and Wagnerand and others [8]. Even ceftriaxone was found to be resistant to hydrolysis by beta-lactamases from *Enterobacter cloacae* and *Bacillus cereus*, but it will not produce inhibitory effect against

B. cereus [14]. According to this property, ceftriaxone seems to be a good selective agent in the *B. cereus* recovery for the samples with high contaminated flora.

Growth recovery of *B. cereus* under different selective pressures from 3 potential inhibitors (including Polymyxin B sulfate, amoxicillin, and ceftriaxone) was studied. *B. cereus* grew fairly well in TSB multiplying from a few cells to 10^7 CFU/mL within 24 h. However, the use of amoxicillin in TSB produced strong negative effect towards *B. cereus* growth. The only promising candidates of selective inhibitors for *B. cereus* recovery was ceftriaxone at 20 mg/mL. For this supplement, ceftriaxone showed good preference towards the growth of *B. cereus* in TSB and significant toxicity against *E. coli* and *S. aureus*. The use of Polymyxin B in TSB, on the other hand, was widely integrated into *B. cereus* selective enrichment medium but did not differentiate the growth of *S. aureus* from *B. cereus*. The information obtained from this research could provide a guideline for decreasing the enrichment step and cost when comparing to the routine protocols which used the expensive media (e.g., Mannitol Yolk Polymyxin B Agar, Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar, Brilliance *Bacillus cereus* Agar). The results from this finding could lead to more efficient enrichment step reducing the overall detection time and lower the cost of analysis.

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References

[1] Ehling-Schulz, M.; Fricker, M, and Scherer, S. (2004). *Bacillus cereus*, the Causative Agent of an Emetic Type of Food-Borne Illness. *Molecular Nutrition & Food Research*. *48*(7), 479-487.

[2] Bottone, E.J. (2010). *Bacillus cereus*, a Volatile Human Pathogen. *Clinical Microbilogy Reviews*. *23*(2), 382-398.

[3] Schlegelova, J.; Brychta, J.; Klimova, E.; Napravnikova, E, and Babak, V. (2008). The Prevalence of and Resistance to Antimicrobial Agents of *Bacillus cereus* Solates from Foodstuffs. *Veterinarni Medicina*. *48*(1), 331-338.

[4] USFDA. (2009). Unilever Conducts Nationwide Voluntary Recall of Slim-Fast® Ready-to-Drink Products in Cans Due to Possible Health Risk. Retrieved from http://www.fda.gov

[5] Tallent, S.M.; Kotewicz, K.M.; Strain, E.A, and Bennett, R.W. (2012). Efficient Isolation and Identification of *Bacillus cereus* Group. *Journal of AOAC International*. *95*(2), 446-451.

[6] Donovan, K.O. (1958). A Selective Medium for *Bacillus cereus* in Milk. *Journal of Applied Microbiology*. *21*(1), 100-103.

[7] Wong, M.H.; Zeng, L.; Liu, J.H., and Chen, S. (2013). Characterization of Salmonella Food Isolates with Concurrent Resistance to Ceftriaxone and Ciprofloxacin. *Foodborne Pathogens and Disease*. *10*(1), 42-46.

[8] Wagner, R.D.; Johnson, S.J.; Cerniglia, C.E., and Erickson, BD. (2011). Bovine Intestinal Bacteria Inactivate and Degrade Ceftiofur and Ceftriaxone with Multiple Beta - Lactamases. *Antimicrobial Agents and Chemotherapy*. *55*(11), 4990-4998.

[9] Qadri, S.M.; Ueno, Y., and Cunha, B.A. (1996). Susceptibility of Clinical Isolates to Expanded-Spectrum Beta-Lactams Alone and in the Presence of Beta-Lactamase Inhibitors. *Chemotherapy*. *42*(5), 334-342.

[10] Fazlani, S.A.; Khan, S.A.; Faraz, S., and Awan, M.S. (2011). Antimicrobial Susceptibility of Bacterial Species Identified from Mastitic Milk Samples of Camel. *African Journal of Biotechnology*. *10*(15), 2959-2964.

[11] Chon, J.W.; Hyeon, J.Y.; Park, J.H.; Song, K.Y.; Kim, J.H., and Seo, K.H. (2012). Improvement of Mannitol-Yolk-Polymyxin B Agar by Supplementing with Trimethoprim for Quantitative Detection of *Bacillus cereus* in Foods. *Journal of Food Protection*. *75*(7), 1342-1345.

[12] Gottfried, M.; Orrego, C.; Keynan, A., and Halvorson, HO. (1979). Specific Inhibition of Outgrowth of *Bacillus subtilis* Spores by Novobiocin. *Journal of Bacteriology*. *138*(2), 314-319.

[13] Abd, A.H.A.; Abood, A.S., and AbdelA'Al, N.I. (2012). Study the Susceptibility of *Bacillus cereus* Isolated from Milk and Milk Products to Antibiotics. *Journal of Kerbala University*. *10*(3), 1-4.

[14] ISO. (2006). Microbiology of Food and Animal Feeding Stuffs-Horizontal Method for the Determination of Low Numbers of Presumptive *Bacillus cereus*- Most Propable Number Tecnique and Detection Method. *International Organization for Standardization*. Geneva.

[15] Khueankhancharoen, J., and Thipayarat, A. (2011). Application of Modified Drop Plate Technique (MDPT) and Logistic Model to Optimize Non-Selective Substrates for *Salmonella* Typhi Resuscitation. *Asian Journal of Food and Agro-industry*. *4*(6), 349-358.

[16] Donovan, K.O. (1958). A Selective Medium for *Bacillus cereus* in Milk. *Journal of Applied Microbiology*. *21*(1), 100-103.

[17] Hermsen, E.D.; Sullivan, C.J., and Rotschafer, J.C. (2003). Polymyxins: Pharmacology, Pharmacokinetics, Pharmacodynamics, and Clinical Applications. *Infectious Disease Clinics of North America*. *17*(3), 545-562.

[18] Turnbull, P.C.; Sirianni, N.M.; LeBron, C.I.; Samaan, M.N.; Sutton, F.N.; Reyes, A.E., and Peruski, L.F. (2004). Mics of Selected Antibiotics for *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, and Bacillus mycoides* from a Range of Clinical and Environmental Sources as Determined by the Etest. *Journal of Clinical Microbiology*. *42*(8), 3626-3634.

[19] Ko, S.Y.; Chung, H.J.; Sung, H.S., and Kim, M.N. (2007). Emergence of Beta-Lactam-Dependent *Bacillus cereus* Associated with Prolonged Treatment with Cefepime in a Neutropenic Patient. *Korean Journal of Laboratory Medicine*. *27*(3), 216-220.