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Note

Development of an improved selective media for differentiation of emetic and diarrheal type *Bacillus cereus*

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Abstract The objective of this study was to develop a differential medium with improved selectivity for the isolation of *Bacillus cereus*. Mannitol egg yolk polymyxin medium supplemented with D-galactose allowed the differentiation of diarrheal- and emetic-type *B. cereus* through pH monitoring. The pH of the medium decreased significantly when incubating the emetic-type *B. cereus*, whereas the pH change was not significant when incubating the diarrheal-type. The addition of pH indicators, such as methyl red and phenol red, to the medium allowed visual differentiation between diarrheal- and emetic-type *B. cereus*. A solid agar medium was also developed by optimizing the concentrations of medium components such as monosaccharides, agar, egg yolk enrichment, pH indicators, and antibiotics. This study indicates the possibility of applying selective media for the differentiation of diarrheal- and emetic-type *B. cereus*.

Keywords: selective media, *Bacillus cereus*, biochemical property, detection, differentiation

Introduction

Foodborne illnesses are commonly caused by *Bacillus cereus*, a bacterium often found in rice-based food and fresh produce. *B. cereus* is a gram-positive, spore-forming foodborne pathogen (Ghosh and Setlow, 2010). Under sporulation conditions, such as a nutrient deficient environments, *B. cereus* cells form a spore-coat and an exosporium layer and significantly decrease the moisture content in the core of the pathogen (Choi and Kim, 2020). Two types of *B. cereus* that cause foodborne illnesses have been reported: diarrheal-type and emetic-type. Illness caused by the emetic-type *B. cereus* is more severe than illness caused by the diarrheal-type due to the difference in toxins produced in food products by the bacteria (Kim et al., 2010). It is important to detect and control these types of pathogens in food products.

Selective and differential media are widely used to isolate foodborne pathogens in food products. However, the sensitivity and selectivity of conventionally used selective and differential media is limited, highlighting the importance of the continued development of media with improved properties (Park et al., 2011). Even though Mannitol Egg Yolk Polymyxin (MYP) medium is widely used to isolate *B. cereus* in food products, it does not differentiate between diarrheal- and emetic-type *B. cereus* (Kwon et al., 2021). Different biochemical properties of diarrheal- and emetic-type *B. cereus* have been reported previously (Hong et al., 2020). Therefore, conventionally used MYP medium may be optimized to differentiate diarrheal- and emetic-type *B. cereus* in food products.

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Materials and Methods

Bacterial cultures and cell suspension

Bacillus cereus strains were used as per a previous publication by Hong et al. 2020 (Table 1). Other pathogens, such as *Bacillus subtilis*, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Listeria innocua*, and *Staphylococcus aureus*, were prepared for selectivity experiments according to the method of Jeong et al. 2020. Briefly, a single colony grown on tryptic soy agar (TSA; Difco, Detroit, MI, USA) was inoculated into 5 mL of tryptic soy broth (TSB; Difco, Franklin Lakes, NJ, USA) and incubated at 30°C for 24 h. After incubation, the bacterial cultures were vortexed and centrifuged for 20 min at 4470×*g*. The resulting pellets were resuspended in 9 mL of 0.2% sterile peptone water (PW; Difco).

Development of selective liquid medium for differentiation of emetic-type *B. cereus*

Guided by the results from a previous study by Hong et al. 2020, a range of substances, shown to have differential fermentation patterns for diarrheal- and emetic-type *B. cereus,* were chosen for the development of a new differential medium. An Analytical Profile Index (API) test was performed and D-galactose supplemented MYP media showed differential growth positive (+) for emetic-type *B. cereus* and negative (−) for diarrheal-type. Consequently, D-galactose (Sigma Aldrich, St. Louis, MO, USA) supplemented liquid media was further investigated. Media were prepared with the composition indicated in Table S1 and 10 mL of each medium was added to a test-tube. After autoclaving at 121°C for 15 min, the media was cooled down to 55°C and the egg yolk enrichment (BD Difco) was added. Bacterial culture (40 µL) was inoculated into 10 mL of each medium. The pH of the inoculated liquid media was determined using a pH meter (ST2100, OHAUS, Parsippany, NJ, USA) before and after pathogen incubation (30°C

for 24 h). Thereafter, pH indicators were chosen, such as methyl red and phenol red, their optimal concentrations were determined before adding them to the media, and the samples were autoclaved.

Development of selective solid medium

Bacto agar (Difco, Becton, Detroit, MI) was added to the medium, prepared with the components listed in Table S1, and dissolved in distilled water under heating and stirring with hot plate and stirrer (MSH-20D, DAIHAN Scientific Co. Ltd, Wonju-si, Gangwon-do, South Korea). The culture medium was cooled to 55°C after autoclaving before addition of the egg yolk enrichment and polymyxin B and the mixture was poured into petri dishes. Spread plating was performed by pipetting 100 µL of various dilutions (serial diluted up to ten-fold in 0.2% PW) of resuspended bacterial culture onto the solid medium to identify the effect of pathogen population (1, 3, and 5 log CFU/mL). Streak plating was performed by streaking out one loopful of bacterial culture onto the prepared agar medium. The characteristics of individual colonies were examined after 24 h of incubation at 30°C.

Adjustment of concentrations of constituents

Concentrations of medium constituents such as monosaccharides, agar, egg yolk enrichment, pH indicator, and antibiotics were optimized. For example, varying amounts of D-galactose (10, 12, 15, 17, and 20 g) were added to 900 mL of the medium. Likewise, the concentrations of Bacto™ agar and egg yolk enrichment were optimized. The concentration of the antibiotic Cefadroxil was adjusted from 1.7% to 0.0002% to detect *B. cereus* selectively and sensitively.

Statistical analysis

Experiments for pH determination were conducted in triplicate and one-way analysis of variance (ANOVA) was performed using the SPSS (IBM SPSS Statistics 26, Chicago, IL, USA). Mean values were assessed using Duncan's multiple comparison test and significant differences were determined at the level of p=0.05.

Results and Discussion

Fermentation properties of diarrheal- and emetic-type *B. cereus* in D-galactose supplemented medium

The pH of the medium, after incubating with either diarrheal- or emetic-type *B. cereus,* was determined to confirm the hypothesis that these bacteria have different fermentation properties for D-galactose supplemented medium. As expected, the pH of the media after incubation was significantly different for diarrheal- and emetic-type *B. cereus* (Table 1). The pH of the media incubated with emetic-type *B. cereus* was between 4.71 and 4.74, whereas media incubated with diarrheal-type *B. cereus* had pH values between 5.77 and 6.11. The result supports our previous findings (Hong et al., 2020) that emetic-type *B. cereus* can ferment D-galactose, whereas diarrheal-type lacks this ability. To visualize the pH changes, pH indicators, such as methyl red and phenol red, were added to the medium. Different colors were observed for emetic-type *B. cereus* incubated medium and diarrheal-type *B. cereus* incubated medium regardless of pH indicators, however, the color change with phenol red was more apparent than that with methyl red. When phenol red was used, emetic-type *B. cereus* incubated medium presented a clear yellow color and diarrheal-type *B. cereus* incubated medium presented an orange color; except for *B. cereus* ATCC 21768, which showed orange/yellow color. In subsequent experiments, *B. cereus* ATCC 21768 also showed intermediate characteristics of diarrheal- and emetic-type *B. cereus*. For the agar media, the diarrheal-type *B. cereus* presented a pink color and the emetic-type *B. cereus* presented a yellow color (Table 2 and Fig. S1). Even though the strains used in this study are limited, the results indicate that D-galactose can be used as a selective material for the differentiation of diarrheal- and emetic-type *B. cereus*. Various studies have been reported for the improvement of selective media for *B. cereus*, but this is the first study that developed an agar medium able to visually detect diarrheal- and emetic-type *B. cereus* selectively (Fricker et al., 2008). The concentrations of medium constituents, such as mono-

Table 1. Color and pH values of developed liquid media (by adding D-galactose and excluding agar from conventionally used Mannitol Egg Yolk Polymyxin Agar) before and after incubation of diarrheal and emetic type *B. cereus*

Type of pathogen	Incubated bacteria strain	pH[1)]	Color
Control		6.86±0.00A	Orange
Diarrheal type *B. cereus*	*B. cereus* ATCC 14579	6.08±0.02BC	Orange
	B. cereus ATCC 21768	5.77±0.03F	Orange+Yellow
	B. cereus ATCC 13061	6.06±0.01CD	Orange
	B. cereus ATCC 10876	6.04±0.01DE	Orange
	B. cereus ATCC 1094	6.11±0.03B	Orange
	B. cereus ATCC 10987	6.04±0.01D	Orange
	B. cereus ATCC 3674	6.01±0.01E	Orange
Emetic type *B. cereus*	*B. cereus* Emetic type (Isolation A)	4.74±0.02G	Yellow
	B. cereus Emetic type (Isolation B)	4.71±0.01G	Yellow
	B. cereus Emetic type (Isolation C)	4.71±0.01G	Yellow

[1)]Mean values standard deviation. Values in the same column followed by the same upper-case letter are not significantly different (p>0.05)

Table 2. Color values of *B. cereus* colonies incubated on developed solid media with adjusted concentrations of constituents

	1.67% D-galactose, 0.55% D-mannitol, and 1.67% agar				1.11% D-galactose, 1.11% D-mannitol, and 1.11% agar			
Bacterial strain	5 log[1)]	3 log	1 log	streaking	5 log	3 log	1 log	streaking
B. cereus ATCC 14579	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink
B. cereus ATCC 13061	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink
B. cereus Emetic A	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
B. cereus Emetic B	Yellow	Yellow	Yellow+Pink	Yellow	Yellow	Yellow	Yellow+Pink	Yellow
B. cereus Emetic C	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow

[1)]Population of initial inoculum (log CFU/ml), Concentrations of other constituents are not different from conventionally used Mannitol Egg Yolk Polymyxin Agar except for egg yolk (2.78%)

Table 3. Color values of microorganisms grown in the developed media with various concentrations of Cefadroxil

	0.67% Cefadroxil			0. 44% Cefadroxil			0.0002% Cefadroxil		
Bacterial strain	5 log[1)]	3 log	1 log	5 log	3 log	1 log	5 log	3 log	1 log
B. cereus ATCC 14579	Pink	ND	ND	Pink	Pink	ND	Pink	Pink	Pink
B. cereus ATCC 21768	ND	ND	ND	ND	ND	ND	Pink	Pink	Pink
B. cereus ATCC 13061	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink
B. cereus Emetic A	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
B. cereus Emetic B	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow+Pink

[1)]Population of initial inoculum (log CFU/mL)

saccharides, agar, egg yolk enrichment, pH indicator, and antibiotics, were optimized to maximize the efficiency of the developed medium.

Application of various antibiotics for selective medium

Various antibiotics, including oxacillin, polymyxin B, lithium chloride, and cefadroxil, were added to the medium to isolate *B. cereus* selectively. When 0.001% oxacillin was added, diarrheal- and emetic-type *B. cereus* grew well—reaching 10^5 CFU/mL—and showed the differential pink- and yellow-colored colonies described above. However, other pathogens, such as *E. coli* O157:H7 and *S. Typhimurium*, still showed growth; displaying only yellow colonies. Therefore, lithium chloride, an antibiotic used to inhibit the growth of gram-negative bacteria, was combined with oxacillin to inhibit the growth of *E. coli* O157:H7 and *S. Typhimurium*. The addition of 0.001% oxacillin and 1.5% lithium chloride was sufficient for inhibiting the growth of *E. coli* O157:H7 and *S. Typhimurium* when inoculating the medium with 1-3 log CFU/ml of these bacterial cultures; however, when inoculating the medium with 5 log CFU/mL of these bacterial cultures, growth was not completely inhibited (data not shown). Next, a combination of polymyxin B and oxacillin was investigated for the inhibition of growth of other pathogens, such as *E. coli* O157:H7, *S.* Typhimurium, *L. innocua*, and *S. aureus* (Table S2). *S.* Typhimurium was shown to grow on the medium with 0.18% polymyxin B and 0.002% oxacillin. When more than 0.22% polymyxin B was added in addition to 0.002% oxacillin, false positive results, from growth of other pathogens, were not observed regardless of the initial inoculation concentration (1-5 log CFU/mL). Unfortunately, this combination of antibiotics at these concentrations also prevented the differentiation of diarrheal- and emetic-type *B. cereus* as the emetic-type *B. cereus* incubated medium also turned pink in color. Cefadroxil, a β-lactam-type cephalosporin antibiotic, was also investigated (Table 3). It has been shown that cefadroxil has an inhibitory effect on the growth of *S. aureus*, one of the microorganisms responsible for the false-positive results mentioned above (Buck and Price, 1977). When using 0.0002% cefadroxil, the differentiation of diarrheal- and emetic-type *B. cereus* was still possible, but false-positives were still observed. Therefore, a two-step approach is necessary to selectively detect diarrheal- and emetic-type *B. cereus*, first using medium containing oxacillin and polymyxin B in combination, followed by medium containing cefadroxil. It is worth noting that several strains of *B. cereus* and non-*Bacillus* strains were used to identify the sensitivity and selectivity of the medium.

Conclusion

In the present study, a selective medium to differentiate diarrheal- and emetic-type *B. cereus* was developed based on differences in their fermentation of D-galactose. The incubation of emetic-type *B. cereus* with the medium developed in this study decreased the pH value significantly, whereas the change was marginal for diarrheal-type *B. cereus*. The concentrations of medium constituents, such as monosaccharides, agar, egg yolk enrichment, pH indicator, and antibiotics, were optimized to maximize the efficiency of the developed medium. Various antibiotics, such as oxacillin, polymyxin B, lithium chloride, and cefadroxil, were investigated to ensure the selectivity of the developed medium. Although the bacterial strains and the results of the present study are limited, this is the first investigation into the development of selective medium for the differentiation of

diarrheal- and emetic-type *B. cereus*. Nevertheless, further studies are needed to overcome the current limitations of the medium developed in this study.

Conflict of Interest

The authors declare no conflict of interest.

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