SPORULATION EFFICIENCY OF CLOSTRIDIUM DIFFICILE IN FOUR PRE-REDUCED, ANAEROBICALLY-STERILIZED BROTH FORMULATIONS

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Abstract

The utility of a commercially-prepared broth formulation for the reliable and reproducible production of *Clostridium difficile* spores facilitates a variety of research applications. To determine which formulation is most suitable for spore production, four Pre-Reduced, Anaerobically-Sterilized (PRAS) broth formulations, including Brain Heart Infusion Broth (BHI), Peptone Yeast Extract Broth (PY), Duncan and Strong Sporulation Broth (DS) and Wilson's Sporulation Broth (SB), were compared for their ability to allow *C. difficile* ATCC 700057 to form spores. The strain was grown in triplicate, in each of the four media, for each time point. Each broth was subcultured —at 24, 48, and 72 hours—and serially diluted to determine the total viable count. The replicates were then heat activated and the vegetative forms killed, by heating at 70° C, for 10 minutes. Serial dilutions were then performed and subsequently plated on two PRAS agars: Brucella Blood Agar (BRU) and Brain Heart Infusion agar with Horse Blood and Taurocholate (BHI-HT). Colony-forming units were then counted and compared for each broth formulation and the ability of BRU and BHI-HT to recover both vegetative and spore forms of *C. difficile*. Wilson's Sporulation Broth (SB) consistently produced the greatest significant quantity of spores recovered on either agar ($P < 0.005$). Brucella Blood Agar (BRU) recovered a higher quantity of total viable cells and spores from the BHI and PY broth formulations as compared to the BHI-HT; however, the BHI-HT agar recovered more total viable cells and spores from the SB and DS broth formulations ($P < 0.005$). Based on this study, it appears that the SB formulation is capable of reproducibly generating the highest number of viable spores from *Clostridium difficile* strain 700057, and that the BHI-HT agar formulation is valuable in optimizing the recovery of total viable cells and heat-treated spores.

Introduction

To determine the broth with the highest capability of Clostridium difficile spore production, four broth formulations were chosen. Two commonly referenced broth formulations DS and SB along with two commercially available formulations PY and BHI were evaluated. The length of time that is necessary to reproducibly create a spore population of greater than 107 spores/mL varies widely in literature from 1 to 7 days (2), (10). A rapid method for the production of spores would facilitate any project requiring the production of spores so 1, 2, 3, and 5 days were evaluated for the greatest quantity of spores produced. The efficiency of the temperature and length of time used to heat activate the spores and kill vegetative cells was not evaluated. To ensure the accurate detection of the spore population two formulations of agar media were chosen to determine germination efficiency. The first formulation is the widely available Brucella Blood Agar, the second is a custom Brain Heart Infusion Agar with Horse Blood and Taurocholate. Horse Blood and Taurocholate have been cited as growth and germination stimulants (8). The BHI formulation base was chosen because of its reported ability to allow production of a high toxin concentration compared to other basal broth formulations (1).

Objectives

- Determine the most reliable and productive broth formulation for the generation of *Clostridium difficile* spores.
- Determine if the formulation of agar used to recover spores affects the recovery efficiency.
- Determine the optimal incubation time of the broth formulation to obtain a high yield of spores.

Materials and Methods

Organisms: The organism used for this study was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Strain tested was: *Clostridium difficile* 700057. The organism was subcultured twice after 24 hours from -70°C freezer stocks onto pre-reduced Brucella Blood Agar (BRU) plates (Anaerobe Systems, Morgan Hill, CA) before being inoculated in triplicate in to each broth formulation. All manipulations of bacteria were performed in an Anaerobe Systems AS-580 anaerobic chamber (Anaerobe Systems, Morgan Hill, CA).

Inoculation: A solution with turbidity equivalent to a 0.5 McFarland was created in 1.0 mL PRAS saline blanks (Anaerobe Systems). Broth formulations were inoculated in triplicate with 10uL of standardized innoculum from 24 hour old Brucella Blood Agar (BRU, AS-111, Anaerobe Systems). All manipulations of bacteria were performed under anaerobic conditions.

Media: The growth media used for this study were Pre-Reduced Anaerobically Sterilized (PRAS) broth formulations including Brain Heart Infusion Broth (BHI), Peptone Yeast Extract Broth (PY), Duncan and Strong Sporulation Broth (DS) and Wilson's Sporulation Broth (SB)—see table 1.0. Seven milliliters of each broth was dispensed under anaerobic conditions into 16 x 100 mm tubes with phenolic screw caps and hungate stoppers. Tubes were terminally sterilized in the autoclave for 15 minutes. Tubes were packaged in foil pouches under anaerobic conditions and stored at room temperature.

Incubation: All broth formulations were incubated at their respective time interval hours (+/-2 hours) at 37°C in an Anaerobe Systems AS-580 anaerobic chamber.

Heat Activation: After each of the four broth formulations had been incubated for their respective time period and sampled for initial total viable count the tubes were placed in a 70°C heat block for 10 minutes (~4 minutes ramp up time). This was done to kill vegetative forms and to heat activate the remaining spores to increase recovery efficiency. Tubes were then cooled to room temperature (~45 minutes) and then sampled and serially diluted to determine the heat viable spore population.

Serial Dilution: The total viable counts and the viable spore populations were determined by serial dilution in 9.0 mL PRAS phosphate buffered dilution blanks AS-908 (Anaerobe Systems). One milliliter of broth was added to 9.0 mL of dilution blank and mixed vigorously. 100 uL of appropriate dilutions were plated in duplicate on both BRU and BHI-HT and spread using sterile glass rods. Plating was performed under anaerobic conditions. Plates were then incubated for 48 hours at 37°C in an Anaerobe Systems AS-580 anaerobic chamber and then counted.

Recovery Agar Formulations: Determination of total viable count was performed by serial dilution and plating in duplicate on PRAS Brucella Blood Agar (BRU) and Brain Heart Infusion Agar with Horse Blood and Sodium Taurocholate (BHI-HT). See Table 1.0 for ingredient list.

Reading: Each of the duplicate plates containing 30 to 300 colonies were visually counted and averaged for the final total.

Discussion:

- SB was the broth formulation that consistently generated the highest yield of spores.
- BHI-HT was better at recovering a higher number of CFU's from SB and DS broths specifically at longer time points.
- BRU was better at recovering a higher number of CFU's from PY and BHI broths. Coincidentally PY and BHI broth formulations both utilize cysteine as a reducing agent.
- 120 hours of incubation of SB had the highest percentage of spores compared to time zero total viable counts; however the greatest total number of spores was obtained after 24 hours of incubation.
- Determine the nature of the variability in the plate counts for future experiments. Evaluate if the lot of broth formulation or post heat cooling time correlates to the variable nature of the plate counts.

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TABLE 1.0 MEDIA COMPOSITION

Amount in grams/Liter

Ingredients	PY	BHI	SB	SBI	SBII	DS	BRU Agar	BHI-HT Agar
Pancreatic Digest of Casein	20.0		90.0	25.0	30.0		10.0	
Yeast Extract	10.0	5.0		15.0		4.0	2.0	5.0
Proteose Peptone		10.0	5.0	5.0	5.0	15.0		10.0
Animal Tissue				25.0	30.0		10.0	
Soy Peptone				25.0	30.0		3.0	
Cystine							0.4	
Cysteine	0.5	0.5						
Dextrose		2.0					1.0	2.0
Agar							15.0	15.0
Sheep Blood (mL)							45.5	
Horse Blood (mL)								70.0
Sodium Taurocholate								1.0
Hemin	0.005	0.005					0.005	0.005
Vitamin K1	0.001	0.002					0.01	0.002
Resazurin	0.001	0.001						0.001
Distilled Water (L)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Calcium Chloride, anhydrous	0.008							
Magnesium Sulfate, anhydrous	0.008							
Potassium Phosphate, monobasic	0.04							
Potassium Phosphate, dibasic	0.04							
Sodium Chloride	0.08	5.0					5.0	5.0
Sodium Bicarbonate	0.4							
Ammonium Sulfate			1.0	1.0	1.0			
Trizma Base			1.5	1.5	1.5			
Starch						4.0		
Sodium Thioglycollate						1.0		
Sodium Phosphate, dibasic		2.5				10.0		2.5
Sodium Bisulfite							0.1	
Tryptophan							0.2	
Calf Brain Infusion		7.7						7.7
Beef Heart Infusion		9.8						9.8
Activated Carbon (chips)						1		

TABLE 2.0 SPORE COUNT

Brucella Blood Agar (BRU)

	TOTAL VIABLE COUNT (CFU)				SPORE COUNT POST-HEAT TREATMENT (CFU)				PERCENT CHANGE (SPORES POST-HEAT / TOTAL VIABLE COUNT)			
	24 AVG	48 AVG	72 AVG	120 AVG	24 AVG	48 AVG	72 AVG	120 AVG	24 AVG	48 AVG	72 AVG	120 AVG
BHI	9.22e+07	3.03E+07	1.45E+07	1.07E+07	3.43E+05	3.63E+05	1.01E+06	4.68E+05	0.37	1.20	6.95	4.38
SB	5.63E+07	3.55E+07	2.97E+07	1.08E+07	8.22E+06	7.88E+06	1.03E+07	1.35E+06	14.59	22.20	34.57	12.50
SB(I)	1.61E+09	8.90E+07	2.24E+08	9.58E+07	3.21E+07	3.05E+06	3.73E+07	3.19E+07	1.99	3.43	16.66	33.23
SB(II)	1.20E+09	4.70E+07	2.46E+08	2.43E+07	4.00E+07	2.17E+06	2.63E+07	1.05E+06	3.34	4.62	10.67	4.33
DS	1.25E+08	6.07E+06	1.23E+06	4.08E+06	1.11E+05	3.62E+04	3.50E+04	3.48E+04	0.09	0.60	2.85	0.85
PY	2.01E+08	1.17E+07	4.28E+06	8.56E+06	3.05E+05	5.40E+05	1.45E+06	4.73E+06	0.15	4.62	33.88	55.21

Brain Heart Infusion Agar with Horse Blood and Taurocholate (BHI-HT)

	TOTAL VIABLE COUNT (CFU)				SPORE COUNT POST-HEAT TREATMENT (CFU)				PERCENT CHANGE (SPORES POST-HEAT / TOTAL VIABLE COUNT)			
	24 AVG	48 AVG	72 AVG	120 AVG	24 AVG	48 AVG	72 AVG	120 AVG	24 AVG	48 AVG	72 AVG	120 AVG
BHI	9.79E+07	1.80E+07	1.09E+07	9.92E+06	3.08E+05	1.92E+05	4.54E+05	2.67E+06	0.32	1.06	4.16	26.89
SB	5.80E+07	6.33E+07	4.01E+07	3.04E+07	1.03E+07	1.28E+07	1.29E+07	1.48E+07	17.67	20.18	32.25	48.75
SB(I)	1.57E+09	7.68E+07	1.71E+08	1.75E+08	2.73E+07	6.82E+06	6.99E+07	3.94E+07	1.73	8.87	40.83	22.45
SB(II)	1.62E+09	4.95E+07	3.15E+08	3.82E+07	3.19E+07	4.02E+06	2.98E+07	8.67E+06	1.97	8.12	9.46	22.67
DS	1.50E+08	8.15E+06	1.60E+06	4.65E+06	1.26E+05	4.50E+04	8.40E+04	4.23E+04	0.08	0.55	5.27	0.91
PY	1.56E+08	1.13E+07	5.43E+06	1.44E+07	3.36E+05	7.63E+05	1.76E+06	5.33E+06	0.22	6.75	32.41	37.04

Percent Difference (BHI-HT/BRU)

	PERCENT DIFFERENCE TOTAL VIABLE COUNT (BHI-HT/BRU)				PERCENT DIFFERENCE SPORE COUNT POST-HEAT TREATMENT (BHI-HT/BRU)			
	24 AVG	48 AVG	72 AVG	120 AVG	24 AVG	48 AVG	72 AVG	120 AVG
BHI	106.19	59.50	75.38	92.82	89.81	52.87	45.14	569.40
SB	102.96	178.32	135.08	280.97	124.75	162.16	126.02	1096.18
SB(I)	97.47	86.33	76.40	182.96	85.02	223.44	187.27	123.57
SB(II)	134.90	105.32	127.91	157.34	79.75	184.97	113.44	824.09
DS	120.53	134.34	129.78	113.88	114.18	124.42	240.00	121.53
PY	77.72	96.58	126.90	168.26	109.94	141.20	121.40	112.88

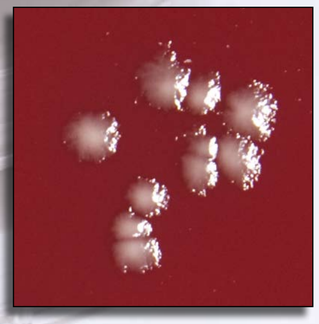
Clostridium difficile **ATCC 700057**

Brain Heart Infusion Agar with Horse Blood & Taurocholate (BHI-HT): 48 Hours

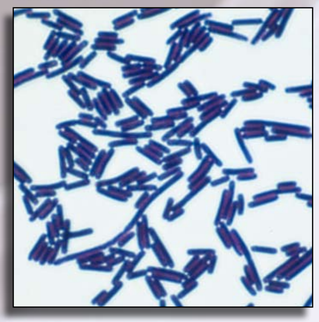
Cycloserine Cefoxitin Fructose Agar (CCFA): 48 Hours

Brain Heart Infusion Agar with Horse Blood & Taurocholate (BHI-HT): 48 Hours Zoom

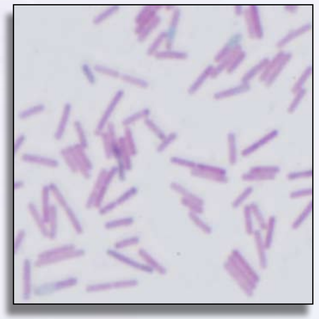
Brucella Blood Agar (BRU): 48 Hours Zoom



Gram Stain 100x oil



Spore Stain 100x oil



Sporulation Broths

1. BHI, 2. DS, 3. PY, 4. SBI, 5. SBII

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