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Evaluation of Recovery Media for Heated *Clostridium sporogenes* Spores[1]

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ABSTRACT

The efficiency of four culture media for recovery of heat-activated and heated *Clostridium sporogenes* spores was studied. Yeast extract agar gave the highest spore recovery. The effect of the method of preparing the yeast extract agar on the recovery of heated spores was also evaluated. The results indicate that (*a*) a significantly lower spore recovery was obtained when the dextrose was omitted completely or when added to the medium before autoclaving, and (*b*) no significant difference in spore recovery was found between yeast extract agar freshly made or prepared and stored at 4 C up to 11 days before use.

Clostridium sporogenes spores are widely used as a heat process test organism in the food and drug industries. Historically, infusion type media have been used for recovery and enumeration of these spores. Preparing infusion media is time-consuming; therefore many workers have attempted to find other equally reliable media for recovering *C. sporogenes* (*1,2,5,6*).

Yeast extract agar (YEA) appears to be a potentially good recovery medium for *C. sporogenes* spores; however, there are several compositional and usage points yet to be resolved. These include the way YEA is prepared (*2,5,6*), the effect of added dextrose and the age of the medium at the time of use.

This study was carried out to determine if YEA can be efficiently used for recovery of *C. sporogenes* spores after heat-activation or for thermally-stressed spores, and to evaluate variation in the preparation and use of YEA, including the effect of adding dextrose to the medium.

MATERIALS AND METHODS

Spore suspensions

Spore suspensions were prepared from *C. sporogenes* PA 3679, obtained from C. F. Schmidt (Continental Can Co., Chicago, Illinois), using Beef Heart Infusion Medium (*1*).

Recovery media

Yeast extract agar (YEA). Yeast extract, 10.0 g; soluble starch, 1.0 g; K_2HPO_4, 2.0 g; agar, 15.0 g; distilled water, 1000 ml; pH 7.2; autoclaved for 15 min at 121 C. Before pouring plates, the following additions were aseptically made to 300 ml of molten medium: 3.75 ml of 10% sodium thioglycollate solution, 3.75 ml of 40% dextrose solution and 7.5 ml of 4% sodium bicarbonate solution. The dextrose solution and the sodium bicarbonate solution were sterilized by membrane filtration, and the sodium thioglycollate solution was sterilized by heat (121 C, 15 min).

Modified YEA. All the YEA medium ingredients, including dextrose, sodium thioglycollate and sodium bicarbonate, were added at the time the medium was prepared. The medium was then autoclaved.

Simplified YEA. Prepared as YEA, except that addition of the 40% dextrose solution at the time of plating was totally eliminated.

Pork infusion agar (PIA). Prepared using the procedure of Stumbo (*4*).

Trypticase soy agar (TSA). Commercial dehydrated medium (BBL) was prepared according to manufacturer's directions.

All media, except the YEA, were made 1 to 11 days in advance of the day of use. The YEA was prepared on the day of use except where indicated.

Heat-activation experiment

In this experiment, the objective was to evaluate the effect of culture media on the recovery of heat-activated spores. Ten µl of the spore suspension containing approximately 10^7 spores per ml were added to a tube containing 10 ml of water for injection (USP)[a]. The tube with its contents was placed in boiling water for 8 min. At the end of the heating time, the tube was transferred to an ice water bath until spore recovery procedures were carried out.

Thermal destruction experiments

In these experiments, the objective was to evaluate the effect of culture media on recovery of thermally-stressed (heated) spores. Five minutes before the start of heating, 1 ml (containing approximately 10^7 spores) of a water suspension of *C. sporogenes* spores was added to an 18 × 150 mm screw-cap test tube containing 10 ml of water for injection (USP). The tube and contents were heated for 3 min in a miniature retort at 121 C in the first, and 7 min at 110 C in the second set of experiments. After heating, the tubes were transferred immediately to an ice water bath until spore recovery was carried out.

Recovery procedures

In a clean room, the heat-activated or the heated spore suspension was diluted in Butterfield's phosphate buffer. Five replicates of the appropriate dilution were pipetted into 100-mm diameter petri plates and about 20 ml of recovery medium was added to each plate. The dilution scheme of the heat-activated or heated spore suspension was chosen on the basis of the number of spores added to the initial tube of water for injection (USP) and the length of the heating time, to give counts in the range of 30 to 300 colonies per plate.

Media that were prepared before the day of use were stored at 4 C. On the day of use the medium was melted by placing it in an autoclave at 121 C for 5 min.

The plates were incubated for 48 h at 32 C in BBL Gaspak anaerobic jars using hydrogen and carbon dioxide Gaspak generators. Colonies were counted with the aid of a Bactronic colony counter.

RESULTS AND DISCUSSION

The average plate counts, the standard deviation and coefficient of variation of colonies recovered using four culture media for heat-activated and heat-stressed *C. sporogenes* spores are presented in Tables 1 and 2 respectively.

The data in Tables 1 and 2 suggest that the recovery of heat-activated spores was of the same magnitude for all

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[a] *Water for injection is water purified by distillation or by reverse osmosis that meets the USP requirements for purified water.*

TABLE 1. *Number of heat-activated*[a] Clostridium sporogenes *spores recovered using four different media.*

Subculture medium	Mean plate count[b] $\bar{N}$	Standard deviation σ	Coefficient of variation $\sigma/\bar{N}$	Percent change from YEA[c]
YEA	43.8	4.32	0.098	
Modified-YEA	48.2	6.72	0.139	+ 10.0
PIA	33.2	5.49	0.165	− 24.2
TSA	38.0	6.78	0.178	− 13.2

[a]Eight minutes at 100 C.
[b]Average of five plates.
[c]Percent change from number of colonies recovered in YEA.

TABLE 2. *Number of heat-stressed*[a] Clostridium sporogenes *spores recovered using four different media.*

Subculture medium	Mean plate count[b] $\bar{N}$	Standard deviation σ	Coefficient of variation $\sigma/\bar{N}$	Percent change from YEA[c]
YEA	191.4	19.77	0.103	
Modified-YEA	47.6	17.03	0.356	− 75.0
PIA	43.2	6.61	0.153	− 77.4
TSA	0.8	0.84	1.046	− 99.6

[a]Three minutes at 121 C.
[b]Average of five plates.
[c]Percent change from number of colonies recovered in YEA.

media tested, whereas the YEA was a better recovery medium for heated spores. These results suggest that the heat-stressed spores have a more specific nutritive requirement than the heat-activated spores, and that the YEA as used in these experiments is the best of the alternatives evaluated.

The PIA described by Stumbo (*4*) does not contain sodium bicarbonate or sodium thioglycollate. Odlaug and Pflug (*3*) reported that addition of these compounds increased recovery of heated *C. botulinum* spores. If these compounds had been added to the PIA in the experiments of this study, the recovery of *C. sporogenes* spores might have been increased.

After YEA was established as a better recovery medium for the heated *C. sporogenes* spores, four yeast extract medium variations were evaluated (Tables 3 and 4).

The number of heat-stressed (3 min, 121 C) *C. sporogenes* spores recovered using fresh and old YEA and fresh and old simplified YEA (addition of 40% dextrose eliminated) is reported in Table 3. In most instances where a comparison is possible, higher counts were obtained when dextrose was added to the medium at the time of plating. The results do not indicate any difference in spore recovery between media prepared the day of the test and media prepared ahead of time.

TABLE 3. *Number of heat-stressed*[a] Clostridium sporogenes *spores recovered as a function of the YEA media type and age.*

Test no.	Recovery medium	Age of medium (Days)	Mean plate count[b] $\bar{N}$	Standard deviation σ	Coefficient of variation $\sigma/\bar{N}$
1	YEA	0	20.0	3.81	0.190
	Simplified YEA[c]	0	5.0	2.34	0.469
	YEA	11	48.2	2.05	0.042
	Simplified YEA	11	21.2	4.02	0.190
2	YEA	0	101.0	9.25	0.091
	YEA	10	60.6	5.32	0.088
	Simplified YEA	10	44.4	6.54	0.147
3	YEA	0	56.2	8.04	0.143
	YEA	10	46.0	6.56	0.143

[a]Heated at 121 C for three minutes.
[b]Mean count of five plates.
[c]Simplified YEA - the addition of 40% dextrose at time of plating eliminated.

TABLE 4. *Number of heat-stressed*[a] Clostridium sporogenes *spores recovered using fresh and old YEA media.*

Tube number	Fresh medium[c]		Old Medium[d]		Difference between log mean count
	Mean plate count[b]	Log mean plate count	Mean plate count[b]	Log mean plate count	(Fresh-Old)
1	118	2.0718	116	2.0644	0.0074
2	118	2.0718	94	1.9731	0.0987
3	95	1.9777	102	2.0086	- 0.0308
4	84	1.9242	84	1.9242	0
5	100	2.0000	101	2.0043	- 0.0043
6	104	2.0170	100	2.0000	0.0170
7	98	1.9912	96	1.9822	0.0089
8	111	2.0453	106	2.0253	0.0200
9	104	2.0170	84	1.9242	0.0927
10	88	1.9444	114	2.0569	- 0.1124
11	102	2.0086	112	2.0492	- 0.0406
		Arithmetic Average of Log Mean Difference			0.0051
		Standard Deviation of Log Mean Difference (σ)			0.0584

[a]Seven minutes at 110 C.
[b]Average of two counts.
[c]YEA prepared on day of plating.
[d]YEA prepared 11 days before plating.

The results of a more extensive experiment comparing the efficiency of fresh and old YEA for the recovery of heat-stressed (7 min, 110 C) *C. sporogenes* spores are shown in Table 4.

The data include the variation in heat destruction (tube to tube) as well as the variation due to media difference (each tube number is a separate independent evaluation). The variation in heat destruction is the difference between the results for the fresh medium and old medium test for each tube number. The results suggest that fresh medium may be better than medium prepared a few days in advance of the test day; however, it appears that the differences are not significant. Since laboratory efficiency increases if media can be prepared in advance, we conclude that this can be done without a significant decrease in the resulting spore recovery.

Yeast extract agar with additives at time of plating is a good recovery medium for heated *C. sporogenes* spores. The results suggest that the basic medium can be prepared in advance and stored at 4 C until used.

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