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Recovery of Clostridia on Catalase-Treated Plating Media

STANLEY M. HARMON* AND DONALD A. KAUTTER
Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204

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Four plating media commonly used for culturing clostridia were tested for their ability to support growth of several *Clostridium* species after storage of the plates for 1 to 10 days at 4 and 25°C with and without subsequent addition of catalase. Liver-veal (LV) agar and brain heart infusion (BHI) agar rapidly became incapable of supporting growth after storage without added catalase, whereas Shahidi Ferguson perfringens agar base and Brewer anaerobic agar were less affected. Plate counts of vegetative cells of nine of the less fastidious *Clostridium* species on untreated LV and BHI agars, stored for 3 days at 4°C, were 60 to 90% lower than counts on catalase-treated media. Counts on Shahidi Ferguson perfringens agar base were only 1 to 24% lower on untreated medium with the same species. Addition of 500 U of purified beef liver catalase to the surface of the 3-day-old agars before inoculation resulted in substantial restoration of the ability of the media to support colony formation from vegetative cells except with the most strictly anaerobic species (nonproteolytic *C. botulinum* types B, E, and F, and *C. novyii* types A and B). A similar response was obtained with spores of the less fastidious species on catalase-treated media. Our results suggest that inhibition of most *Clostridium* species on LV and BHI agars may be due to accumulation of peroxide during preparation, storage, and incubation of the media, and also suggest that the presence of glucose in these media is a major factor contributing to their inability to support growth. It is believed that the addition of exogenous catalase prevents the accumulation of peroxide(s), thus allowing colony formation from vegetative cells of the clostridia under what would otherwise be unsuitable cultural conditions.

The results of an earlier study conducted in our laboratory showed that treatment of several plating media recommended for culturing *Clostridium perfringens* with preparations containing catalase resulted in improved growth and substantial increases in colony counts obtained with this organism (5). Although the stimulatory effect of preparations containing catalase on the growth of anaerobes has been recognized for a long time, the reasons for their effectiveness have not been clearly established. McLeod and Gordon (7) postulated that incorporating catalase into the culture medium prevents the accumulation of H_2O_2 during metabolism of the organism when oxygen is present, and thus inhibition of organisms that are extremely sensitive to peroxide is prevented (2). The production of small quantities of H_2O_2 by respiring cells of some *Clostridium* species has been demonstrated, but the methods used were generally too insensitive to detect the minute quantities of peroxide that inhibit some anaerobes (4). In a previous study with *C. perfringens* (5), we confirmed the finding of Holman (6) that growth of *C. perfringens* in a medium containing glucose is enhanced by adding beef liver extract containing active catalase. It has also been reported that crystalline beef liver catalase improves the growth response of peroxide-sensitive strains of *C. novyii* type B on a plating medium (11). It should be noted, however, that Mateles and Zuber claimed that crystalline catalase had no effect on the growth of *Clostridia* when air was present (8). A second possibility is that addition of exogenous catalase to the culture medium prevents the inhibition of peroxide-sensitive organisms by destruction of H_2O_2 or its complexes which form during preparation and storage of the medium. This effect has been reported for several bacteria, including *Shigella dysenteriae* (9), *Mycobacterium tuberculosis* (1), and *Staphylococcus aureus* (13).

Results of a previous study in our laboratory showed that vegetative cells and spores of *C. perfringens* were inhibited on plating media containing glucose, especially if the plating media were stored in air for several days before use. This effect was largely prevented by treating the medium surface with catalase before

inoculation of the plates.

The purpose of the present study was to identify factors that contributed to the loss of growth-promoting potential of plating media used for culturing clostridia and to determine, if possible, the reasons for the beneficial effect of treating the medium with catalase.

MATERIALS AND METHODS

Organisms and cultural conditions. All cultures used were from the stock culture collection of the Food and Drug Administration. Stock cultures were maintained at room temperature in a glucose-peptone-Trypticase-beef infusion medium (GPTBI) described by Eklund et al. (3) and were subcultured once a week. Test cultures were grown in GPTBI by inoculating 15 ml of freshly steamed and cooled medium with 0.1 ml of stock culture and incubating the inoculated medium at 30°C for 18 to 24 h, depending on the strain tested. This procedure usually provided test cultures which consisted almost entirely of vegetative cells. Spore stocks of cultures other than *C. perfringens* were obtained by incubating GPTBI cultures for 3 to 4 days at 30°C and filtering the culture through sterile cotton. The sporulated cultures were sedimented by centrifugation, washed twice with sterile distilled water, and stored at 4°C until used. Spore stocks of *C. perfringens* were grown in the polypeptone-yeast extract-starch medium described by Tsai et al. (12). Spores of *C. botulinum* types A and B and *C. sporogenes* were heat-activated in a water bath for 20 min at 65°C before the plates were inoculated. No heat treatment was used for spores of the *C. perfringens* and *C. bifermentans* strains tested because they are sensitive to heat.

Plating media. All plating media used, with the exception of modified Shahidi Ferguson perfringens (SFP) agar base, were prepared from commercially available dehydrated media. The liver-veal (LV) agar, Brewer anaerobic (BA) agar without glucose and indicator, and SFP agar base were obtained from Difco. The brain heart infusion (BHI) agar was supplied by BBL. Each medium was prepared and sterilized according to the manufacturer's instructions. The sterilized media were cooled at 50°C in a water bath, and 15 to 20 ml was dispensed into sterile plastic petri dishes (15 by 100 mm). Unless otherwise indicated, the plate surfaces were allowed to dry for 24 h at room temperature and the media were stored in air at 4°C until used. Interpretation of results was simplified by omitting the antibiotics and egg yolk emulsion suggested for use in SFP agar and certain other medium components, such as blood and egg yolk emulsion, usually recommended for use in plating media for culturing clostridia.

Colony counts. To determine the response of vegetative cells on treated and untreated plates, 18- to 24-h GPTBI cultures were diluted in 0.1% peptone-water to give an estimated 1,000 to 3,000 viable vegetative cells/ml, and 0.1 ml of the diluted culture was spread evenly on the surface of duplicate plates that had been treated with catalase or left untreated as controls. The inoculated plates were allowed to dry for a few minutes and then were placed in GasPak anaerobic jars (BBL); anaerobic conditions were established with disposable H_2 and CO_2 gas generator envelopes. To maximize the effects of catalase treatment, plates inoculated with *C. perfringens* were incubated in Case anaerobic jars (Pfizer Diagnostics, Chicago, Ill.) under a nitrogen atmosphere during some experiments. The inoculated plates were incubated at 35°C for 24 or 48 h, depending on the organism, and colony counts were made with a Quebec colony counter. The relative sizes of colonies obtained were also noted in some experiments.

Catalase treatment. A filter-sterilized solution of catalase preparation of the desired concentration in physiological saline was freshly prepared from lyophilized material or from a sterile stock solution of catalase obtained from the supplier. The catalase solutions were diluted in sterile physiological saline, and 0.1 ml was spread evenly on the surface of the previously poured plates with a sterile glass rod 10 min before inoculation of the plates. The total amount of catalase to be added was calculated from information provided by the supplier on a unit-per-milligram basis, and the test solutions were diluted accordingly before use. To simplify the presentation of results, the different preparations were identified by a capital letter. The preparations used and the suppliers were as follows: (A) lyophilized partially purified beef liver catalase (BLC) containing 3,100 U/mg (Calbiochem, Los Angeles, Calif.), (B) purified BLC solution containing 50,000 U/mg (Nutritional Biochemicals Corp., Cleveland, Ohio), and (C) twice-crystallized BLC solution containing 30,000 U/ml (Sigma Chemical Co., St. Louis, Mo.). The concentrated sterile solutions were occasionally stored for a few days at 4°C for use in later experiments.

Effect of heating catalase preparations. To determine the effect of heating on their effectiveness in promoting growth, the catalase preparations were heated at 60 to 65°C. The sterile BLC preparation B was diluted in physiological saline to a concentration of 2,500 U/ml, and a portion was heated in a water bath at 65°C for 1 h. Identical plates of LV agar stored for 24 h after preparation were treated with 0.1 ml of the heated and unheated preparations 10 min before inoculation with the diluted GPTBI cultures. To determine the effect of treating plates with BLC before inactivating the enzyme, LV agar plates were treated with catalase and held at room temperature for 20 min. The plates were placed in plastic bags to prevent dehydration and heated in an air incubator at 60°C for 1 h to inactivate the BLC. The plates were cooled to room temperature and inoculated. Identically treated control plates were stored in air at 25°C.

Effect of carbohydrates. The effect of carbohydrates on recovery with untreated and catalase-treated plates was determined by adding sterile glucose or sucrose to modified SFP agar base after sterilization to give a final concentration of 0.5%. The modified SFP agar base consisted of 1.5% tryptose (Difco), 0.5% yeast extract, 0.5% Soytone (Difco), and 2% agar.

Preincubation in GasPak jars. To determine the effect of preincubation on recovery, the freshly plated media were placed in GasPak jars, and anaerobic conditions were established with disposable H_2 and CO_2 generator envelopes. Media preincubated for 18 h were held in an air incubator at 30°C; those stored for longer periods were held at 25°C.

Effect of added hydrogen peroxide. The effect of added H_2O_2 on recovery with untreated and catalase-treated plates was determined as follows. A 30% H_2O_2 solution was serially diluted from 10^{-4} to 10^{-7} with sterile distilled water, and 0.1 ml was spread on the surface of each plate. The plates were stored in air in a glass jar or placed in a GasPak jar under anaerobic conditions within 20 min after treatment with H_2O_2.

Effect of UV light on media. To determine the effect of ultraviolet (UV) exposure on the media, the plates were irradiated with short- or long-wave UV light in a Chromato-Vue box (Ultra-Violet Products, Inc., San Gabriel, Calif.) at an average distance of 10 cm for 1 h at 25°C. The irradiated media were then either inoculated directly with a diluted GPTBI culture of the test organism or treated with catalase before inoculation as previously described and were incubated in GasPak jars.

RESULTS AND DISCUSSION

The results of preliminary experiments indicated that the response of many *Clostridium* species on plating media treated with catalase was similar to that reported earlier with *C. perfringens* (5). The effectiveness of catalase treatment in promoting growth depended on several factors, including (i) the composition of the particular medium used, (ii) the time and conditions of storage of the medium before use, and (iii) the sensitivity of the vegetative cells of a particular species to exposure in air.

Effect of catalase treatment. Table 1 shows the results obtained with identical vegetative-cell inocula of nine different *Clostridium* species on untreated and catalase-treated LV, SFP agar base, and BHI plates. The dilution factor column in Table 1 indicates the dilution of the GPTBI culture used that yielded countable plates. The media were stored for 10 days in air at 4°C before use and were inoculated by spreading 0.1 ml of an 18-h GPTBI culture diluted in freshly steamed and cooled 0.1% peptone-water. As can be seen in Table 1, higher recoveries were always obtained with catalase-treated plates. Recovery of most species increased substantially when media containing glucose were treated with catalase, and colony size increased considerably on plates treated with 500 U of purified BLC 10 min before inoculation. Colony counts on catalase-treated plates of SFP agar base were higher than those on the untreated plates, but the increased

TABLE 1. *Effect of treating plating media with beef liver catalase on the percent recoveries obtained with vegetative-cell cultures of 10 Clostridium species*[a]

Organism	Dilution factor	Percent recovery[b] on					
		LV agar		BHI agar		SFP agar base	
		Untreated	Treated[c]	Untreated	Treated	Untreated	Treated
C. perfringens FD-1	10^7	6.9	98.9	74.7	100	80.4	99.5
C. bifermentans FDA	10^6	2.1	100	6.1	98.5	95.4	97.4
C. sporogenes WR-17	10^6	1.7	76.7	16.6	98.4	81.6	100
C. botulinum A 62A	10^6	<0.6	79.9	29.2	100	—[d]	—
C. botulinum B 169B	10^6	7.4	59.4	1.9	100	—	—
C. botulinum E Beluga	10^3	15.5	33.1	21.0	31.5	22.7	100
C. botulinum B 2B	10^3	<0.6	10.7	<0.6	30.3	2.8	100
C. botulinum F 202F	10^3	<0.7	1.5	<0.7	<0.7	<0.7	100
C. histolyticum KA 12	10^6	2.4	75.3	12.6	99.7	75.7	100
C. butyricum WR-1	10^6	39.9	82.9	74.2	98.2	99.1	100
C. tertium WR-38	10^6	8.0	92.5	75.4	97.8	91.2	100
C. novyii A WR-3	10^4	<0.2	<0.2	<0.2	100	<0.2	<0.2
C. novyii B WR-4	10^4	<1.2	<1.2	<1.2	62.5	<1.2	100
C. acetobutylicum FDA	10^5	<1.4	<1.4	5.8	100	<1.4	27.6
C. tetani WR-29	10^6	0.4	97.6	13.3	100	91.3	91.5

[a] Plates were surface-inoculated with 18-h GPTBI cultures diluted in freshly steamed and cooled 0.1% peptone-water and were incubated at 32 or 35°C, depending on the organism tested.

[b] Compared with highest colony counts obtained with catalase-treated plates of any medium. Counts ranged from 0 on untreated plates to between 1 and 400 on catalase-treated plates.

[c] A 0.1-ml volume of solution containing 500 U of purified beef liver catalase was spread evenly on the surface of each plate 10 min before inoculation.

[d] No data available.

counts were less than with the other two media. This effect had been shown earlier in experiments with *C. perfringens* and was attributed to differences in composition of the media. The data presented also show that catalase treatment was relatively ineffective for promoting growth from vegetative cells of the more strictly anaerobic species when the surface-inoculating technique was used. Based on previous results obtained by a most probable number method, one may reasonably assume the viable population of the more fastidious species tested to be between 10^8 and 10^9 per ml. Therefore, the low plate counts obtained with these species suggest that the colonies that did develop may have been derived from a small number of spores present in the cultures. Although no attempt was made to determine whether spores were actually present, we have found (unpublished data) that no growth of these species occurs on the media tested unless spores are present in the inoculum. Therefore, we concluded that this cultural procedure was inappropriate for vegetative cells of these species, and these organisms were not included in subsequent experiments. However, when colonies did develop, the recovery obtained was usually greater on catalase-treated plates than on untreated plates. This suggests that recovery of spores of the more strictly anaerobic *Clostridium* species may also be improved by catalase treatment of media, as had been shown previously with *C. perfringens* (5).

Effect of catalase concentration. The effectiveness of different concentrations of a purified BLC (preparation B) was determined on BHI agar plates that had been stored at 4°C for 12 days before use. The data (Table 2) show that vegetative cells of the less fastidious *Clostridium* species tested, such as *C. bifermentans* and *C. sporogenes*, responded in a manner similar to *C. perfringens* on plating media treated with various concentrations of BLC before inoculation. Relative percent recoveries increased gradually with increasing amounts of BLC until a maximal effect was obtained with all three species on plates treated with 1,000 to 2,000 U of BLC.

Effect of storage conditions. Table 3 shows the effect of storing four different plating media for 1 and 7 days in air and for 8 days in GasPak anaerobic jars on the recovery obtained with vegetative cells of seven different *Clostridium* species on untreated and catalase-treated plates. The ability of the LV and BHI agars to support quantitative recovery declined rapidly during storage of the media in air. Within 24 h after plating, relative recoveries on untreated

TABLE 2. *Effect of treating BHI agar with different concentrations of beef liver catalase on percent recoveries obtained with vegetative cells of three Clostridium species*[a]

Catalase added/plate[b] (U)	Percent recovery[c] of		
	C. perfringens	*C. bifermentans*	*C. sporogenes*
2,000	100	100	85.4
1,000	85.2	93.0	100
250	73.5	89.1	93.2
50	66.4	75.2	81.2
10	72.8	25.6	3.9
1	34.9	3.1	1.3
0	9.3	<1	12.5

[a] Plates were stored at 4°C for 12 days before use.

[b] A 0.1-ml volume of sterile saline containing the amount of purified beef liver catalase (preparation B) indicated was spread evenly on the surface of each plate 10 min before inoculation.

[c] Compared with highest colony counts obtained on catalase-treated plates. Counts ranged from 48 with *C. sporogenes* on untreated control plates to 383 on plates treated with 1,000 U of beef liver catalase. Similar results were obtained with the other two species.

LV agar plates ranged from 1% or less with *C. botulinum* types A and B to an above-average value of 28.3% with *C. tertium*. Recovery increased markedly on LV and BHI agar plates treated with catalase; with most species recovery increased to a lesser extent on catalase-treated SFP base and BA agars. The highest recoveries with four of the seven species tested were obtained on BA agar plates treated with BLC. Storage of LV agar plates in GasPak jars for 8 days at 25°C resulted in only a slight increase in recoveries with untreated plates. In most cases, storage of SFP agar base in GasPak jars for 8 days resulted in a decrease in the relative percent recoveries obtained with catalase-treated plates compared with counts on identical control plates stored in air at 4°C. The reason for this effect was not determined, but it may have been related to prolonged storage of media at room temperature or to a change in pH of the medium because of the presence of carbon dioxide in the GasPak jars. This aspect was not investigated further because the presence of excessive moisture and other factors were considered to render this method of storage impractical. However, storage of SFP agar base plates in GasPak jars resulted in a slight improvement in recovery on untreated plates compared with counts on plates stored in air for the same period.

These results indicate that quantitative recovery of most species could not be expected on

TABLE 3. *Effect of storage conditions on percent recoveries obtained with vegetative cells of eight Clostridium species on untreated and catalase-treated plating media*[a]

Organism	Storage conditions		Percent recovery[c] on							
			LV agar		BHI agar		SEP agar		BA agar	
	Atmosphere	Time (days)[b]	Untreated	Treated[d]	Untreated	Treated	Untreated	Treated	Untreated	Treated
C. perfringens	Air	1	1.8	97.2	9.4	99.3	40.2	100	18.5	81.8
	Air	7	<0.4	93.8	12.6	100	3.2	89.8	–	–
	GasPak jar	8	13.9	100	–[e]	–	85.7	65.0	79.3	94.3
C. bifermentans	Air	1	12.7	66.0	3.3	72.9	51.3	100	28.8	62.1
	Air	7	4.7	62.4	41.5	100	50.4	94.0	–	–
	GasPak jar	8	6.4	21.8	–	–	96.2	100	17.3	21.8
C. botulinum A 169B	Air	1	1.2	91.1	12.7	80.2	34.5	96.4	16.9	100
	Air	7	0.6	90.5	5.8	100	35.6	96.7	–	–
	GasPak jar	8	<0.3	40.9	–	–	90.4	54.5	79.8	100
C. botulinum B	Air	1	<1	36.0	2.0	93.0	33.0	38.0	46.0	100
	Air	7	<0.6	25.6	<0.6	93.8	71.3	100	–	–
	GasPak jar	8	<0.3	3.5	–	–	1.4	1.2	87.5	100
C. sporogenes	Air	1	7.7	49.2	23.8	90.1	20.5	36.2	95.7	100
	Air	7	<0.3	60.1	13.8	90.5	49.4	100	–	–
	GasPak jar	8	4.6	56.4	–	–	6.6	4.6	72.8	100
C. tetani	Air	1	24.7	100	81.8	85.4	70.5	86.2	84.8	99.5
	Air	7	10.9	100	8.8	82.3	74.6	76.7	–	–
	GasPak jar	8	50.0	66.6	–	–	93.3	76.1	86.7	100
C. butyricum	Air	1	20.5	80.8	30.1	100	42.3	79.3[f]	47.6	48.4
	Air	7	1.6	74.7	26.6	100	69.0	64.9	–	–
C. tertium WR-2	Air	1	28.3	71.4	55.0	74.4	95.4	100	34.3	34.3
	Air	7	4.8	100	61.4	100	98.6	91.0	–	–

[a] Plates were inoculated with dilute GBTBI culture containing approximately 100 to 400 vegetative cells and were incubated in GasPak jars at 37°C for 24 to 48 h, depending on the organism.

[b] Plates stored for 1 day were held in air at 25°C, and those stored for 7 days were held in air at 4°C. Plates stored for 8 days were held in GasPak jars at 25°C.

[c] Compared with highest colony counts obtained with catalase-treated plates of any of the media.

[d] A 0.1-ml volume of solution containing 500 U of beef liver catalase (preparation A) was spread evenly on the surface of each plate just before inoculation.

[e] No data available.

[f] Poor growth on SFP base and BA agars.

untreated plates of the four media tested, especially those containing glucose, after 1 week of storage in air or in GasPak jars. However, when the plates were treated with an active catalase preparation or when some other precaution was taken to restore growth-promoting ability, the recovery was essentially quantitative. Results of the earlier study conducted in our laboratory with *C. perfringens* indicated that preincubation of plated media in GasPak jars at 30°C for 18 h before inoculation could be used as an alternative to catalase treatment for restoring growth-promoting ability of media. However, the highest counts were always obtained when the two treatments were combined (5).

Effect of preincubation in GasPak jars and treatment with peroxide. The effect of treating BHI and BA agar plates with dilute H_2O_2 solutions and storage of the plates containing peroxide in GasPak anaerobic jars or in air for 20 h at 30°C on percent recoveries obtained with *C. perfringens* is shown in Table 4. Treating plates to which toxic levels of H_2O_2 had been added with 1,500 U of BLC (preparation A) 10 min before inoculation with vegetative cells of *C. perfringens* decreased the inhibitory effect significantly. Preincubation in GasPak jars without subsequent treatment with catalase also resulted in substantial increases in colony counts. These results suggest either that H_2O_2 was inactivated while the plating media were held in GasPak jars overnight at 30°C or that this treatment reduced the inhibitory effect of added peroxide in some other way. Treatment of BHI and BA agar plates with 1,500 U of BLC allowed development of *C. perfringens* colonies from vegetative cells whether the medium was stored in air or in GasPak jars under anaerobic conditions. This finding is consistent with the concept that inhibition of the organisms may be due to accumulation of peroxide(s) (8). The fact that toxicity of the peroxide-treated plating media was relieved by preincubation of the media in GasPak jars could explain why colony counts of *C. perfringens* vegetative cells increased substantially on LV agar and BHI plates preincubated for 24 h in GasPak jars before inocula-

TABLE 4. *Effect of preincubation in GasPak jars and catalase treatment on percent recovery of C. perfringens on plating media containing low levels of added peroxide*[a]

Concn of H_2O_2 added (μg/ml)	Percent recovery[b] on							
	BHI agar				BA agar			
	Stored in air		Prereduced in GasPak		Stored in air		Prereduced in GasPak	
	Untreated	Treated[c]	Untreated	Treated	Untreated	Treated	Untreated	Treated
30	43.7	52.8	–[d]	65.6	4.3	91.8	80.1	98.0
3.0	11.7	61.4	69.4	94.8	5.5	84.1	93.0	96.5
0.3	31.5	59.8	100	102	5.1	78.1	94.1	99.6
0.03	27.6	75.3	91.8	75.8	48.8	83.2	99.1	95.8
0	45.8	88.0	46.4	–	37.5	83.9	100	–

[a] A 0.1-ml volume of H_2O_2 of the indicated strength was spread evenly on the surface of each plate, and the plates were held at room temperature in the air or preincubated in GasPak jars at 30°C for 20 h before inoculation with strain FD-1.

[b] Compared with colony counts obtained with catalase-treated control plates preincubated in GasPak jars. Counts ranged from 11 on untreated BA agar to which 0.1 ml of 0.003% H_2O_2 was added to 343 on BHI agar incubated in GasPak jars and treated with catalase.

[c] Each plate was treated with 1,500 U of beef liver catalase 10 min before inoculation.

[d] No data available.

tion. Recovery of this organism was usually increased further by treating media with BLC before inoculation.

Effect of catalase treatment on recovery of spores. Table 5 shows the recoveries of spores of five *Clostridium* species after catalase treatment of LV agar and SFP agar plates. Percent recoveries of spores of all five species increased considerably on LV agar as a result of catalase treatment. Percent recoveries also increased on SFP agar base plates treated with BLC, but the increase was much less than that for LV agar. The higher recovery of *C. perfringens* spores obtained on untreated LV agar plates in the present study could have been due to incubation of the plates in GasPak jars rather than in Case anaerobic jars. As shown previously, the presence of dissolved oxygen in media is evidently a principal contributing factor in the inability of media to support colony formation. Therefore, it seems likely that higher recoveries of *C. perfringens* spores on untreated plates in GasPak jars were due to rapid reduction of oxygen levels by hydrogen, which does not occur in Case anaerobic jars containing nitrogen. These data show that, although catalase treatment of LV agar does result in some increase in colony counts of spores, the increases are small when compared with results obtained with vegetative cells. Recovery also increased on catalase-treated plates of SFP agar base that had been stored for several days in air at 4°C.

Effect of carbohydrates. The addition of either glucose or sucrose to modified SFP agar base at a final concentration of 0.5% had a very adverse effect on the growth-promoting ability of this medium (Table 6). Recoveries with three different *Clostridium* species declined from maximal levels on catalase-treated plates of SFP agar base to 1% or less on the modified basal medium to which glucose or sucrose was added. These results indicate that the presence of a fermentable carbohydrate in plating media was probably the most important factor contributing to the loss of growth-promoting ability of untreated media. Data presented in Tables 1, 2, and 3 suggest that this effect also occurs with LV agar and BHI agar that contain glucose, as we demonstrated earlier with *C. perfringens* (5). These results differ from the findings of Schroder and Busta (10), who reported that only glucose and mannose were inhibitory for

TABLE 5. *Effect of catalase treatment on percent recovery of spores of five Clostridium species*[a]

Organism tested	Percent recovery[b] on			
	LV agar		SFP agar base	
	Untreated	Treated[c]	Untreated	Treated
C. perfringens	54.7	93.2	70.1	90.7
C. bifermentans	67.1	100	86.5	85.3
C. botulinum A.....	68.9	95.3	84.4	100
C. botulinum B 169B.	31.3	100	95.6	83.8
C. sporogenes	21.9	100	90.5	93.2

[a] Spore stocks were heat-activated for 20 min at 65°C before inoculation.

[b] Compared with highest colony count obtained on catalase-treated plates. Plates media were stored in the dark at 25°C for 4 days before use.

[c] A 0.1-ml volume of solution containing 500 U of partially purified beef liver catalase (preparation A) was spread evenly on the surface of each plate 10 min before inoculation.

C. perfringens in a liquid sodium caseinate medium.

Effect of daylight. Exposing plates of LV and SFP agar base to daylight adversely affected recoveries with vegetative-cell cultures of the three *Clostridium* species tested (Table 7). These three species were the only ones tested for this effect because they appeared to be representative of those species least affected by exposure to air. Recovery of *C. perfringens* from untreated plates of LV agar declined from 39.3% to less than 0.4% as a result of exposing plates to diffuse daylight for about 8 h on a January day. Results obtained with an identical set of plates stored in the dark under similar conditions to serve as controls show that recovery of *C. bifermentans* and *C. sporogenes* as well as *C. perfringens* declined on untreated LV agar plates exposed to daylight. A slight decrease in recovery was also obtained on untreated plates of SFP agar base exposed to daylight. The adverse effect of exposing plating media to daylight on growth of clostridia was mentioned by McLeod and Gordon (7), but these investigators did not present any quantitative data.

Effect of UV irradiation. The ability of untreated plates of LV, SFP base, and BHI agars to support growth with identical vegetative cell inocula of three *Clostridium* species declined considerably as a result of exposing the media to short-wave UV irradiation for 1 h at 25°C

TABLE 6. *Effect of adding carbohydrates to modified SFP agar base on the percent recoveries obtained with three Clostridium species on untreated and catalase-treated plates*[a]

Organism	Percent recovery[b] on					
	SFP agar base		Modified SFP agar base[c]			
			Plus glucose		Plus sucrose	
	Untreated	Treated[d]	Untreated	Treated	Untreated	Treated
C. perfringens NCTC 8238 ..	93.9	100	<0.4	95.4	<0.4	89.7
C. bifermentans	77.5	100	<0.3	90.8	1.3	94.6
C. sporogenes	97.3	99.2	<0.3	67.0	<0.3	100

[a] Inoculated with 0.1 ml of an 18-h GPTBI culture diluted in 0.1% peptone-water. Plates were stored at 4°C for 4 days before use.

[b] Compared with highest colony counts obtained on catalase-treated plates of any medium. Counts ranged from 0 on untreated plates of modified SFP agar with carbohydrate added to about 300 on the catalase-treated plates.

[c] Same as SFP agar base except that ferric ammonium citrate and sodium(*meta*)bisulfite were omitted. A sterile solution of glucose or sucrose was added after sterilization to give a final concentration of 0.5%.

[d] Plates were catalase-treated by spreading 0.1 ml of sterile solution containing 500 U of purified beef liver catalase (preparation B) evenly on the plate surface 10 min before inoculation.

TABLE 7. *Effect of exposing media to daylight on percent recoveries obtained with three Clostridium species on untreated and catalase-treated plates of LV agar and SFP agar base*[a]

Organism	Percent recovery[b] on							
	LV agar				SFP agar base			
	Stored in dark		Exposed to daylight[c]		Stored in dark		Exposed to daylight	
	Untreated	Treated[d]	Untreated	Treated	Untreated	Treated	Untreated	Treated
C. perfringens .	39.3[e]	100	<0.4	89.9	79.8	84.1	81.1	86.3
C. bifermentans	<1.0	88.3	<1.0	92.7	46.2	100	26.2	97.3
C. sporogenes ..	5.3	52.7	<0.6	33.5	51.1	89.4	18.6	100

[a] Plated media were stored in the dark for 3 days at 25°C before use.

[b] Compared with colony counts obtained with catalase-treated plates. Counts of *C. perfringens* on LV agar ranged from 0 on untreated plates to 262 on catalase-treated plates stored in the dark. Similar results were obtained with the other two organisms.

[c] Plates were exposed to daylight near a closed window for approximately 8 h. Control plates were stored in the dark under similar conditions.

[d] A 0.1-ml volume of sterile saline containing 500 U of purified beef liver catalase (preparation B) was spread evenly on the surface of each plate 10 min before inoculation.

[e] Colony size on untreated plates was less than one-third of those on identical plates treated with catalase.

TABLE 8. *Effects of UV irradiation and catalase treatment of plating media on percent recoveries obtained with three Clostridium species*[a]

Organism	Percent recovery[b] on											
	LV agar		Irradiated[c] LV agar		BHI agar		Irradiated BHI agar		SFP agar base		Irradiated SFP agar base	
	Untreated	Treated[d]	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
C. perfringens	6.6	85.6	1.2	87.3	70.3	98.3	27.4	100	54.4	99.1	11.4	98.2
C. bifermentans	<0.4	89.1	0.6	91.8	13.2	96.5	0.6	100	5.5	98.3	38.1	89.5
C. sporogenes ...	<0.3	99.2	<0.3	98.6	9.5	96.7	<0.3	99.7	63.7	100	57.5	84.3

[a] Plates were stored in the dark at 25°C for 18 h before use.

[b] Compared with highest colony counts obtained with vegetative cells on catalase-treated plates of any medium. Counts obtained with *C. perfringens* ranged from 0 on untreated control plates to 347 with catalase-treated SFP agar base.

[c] Plates were exposed to short-wave UV irradiation at a distance of 15 cm for 1 h at 25°C.

[d] A 0.1-ml volume of beef liver catalase (preparation A) containing 5,000 U/ml was spread evenly on the surface of each plate 10 min before inoculation.

(Table 8). Recovery of *C. bifermentans* and *C. sporogenes* vegetative cells on BHI agar declined by a factor of about 10 on irradiated plates compared with results on unirradiated control plates. As these data show, treatment of the plates with BLC 10 min before inoculation restored the growth-promoting potential of both the UV-irradiated and control plates of all three media to near optimal levels. These results suggest that loss of growth-promoting ability of the media was due, at least in part, to peroxide(s) that formed spontaneously in the media or was produced as a result of UV irradiation of the plates. This seems likely because the formation of peroxide(s) under these conditions has been demonstrated in studies with other microorganisms (9, 12). This conclusion is strengthened by the fact that treatment of the toxic media with purified BLC was sufficient to restore essentially all of the growth-promoting ability lost during storage or exposure to UV irradiation (Table 8).

Effect of heated catalase. Table 9 shows the effect of heating purified BLC (preparation B) at 65°C for 1 h and of heating catalase-treated LV agar plates for 1 h at 60°C on recoveries obtained with vegetative cells of three *Clostridium* species. When BLC was heated before treatment of LV agar, the preparation was essentially ineffective for promoting growth of these organisms. Heating plates to which 250 U of BLC had been added for 1 h at 60°C also resulted in a considerable reduction in colony counts obtained with vegetative cells of all three *Clostridium* species. These results suggest that, for treatment to be fully effective, catalase must be present in an active form during incubation of the inoculated plates. The slight increases in recovery obtained on catalase-treated plates may have been due to slight residual activity of BLC or to purging of oxygen from the medium during treatment of the plates at 60°C.

TABLE 9. *Effect of heating a beef liver catalase (BLC) preparation for 1 h at 65°C on percent recoveries obtained with three Clostridium species on LV agar treated with the heated preparation*

Organism	Percent recovery[a] on plates		
	Untreated control	Treated with heated BLC[b]	Heated plates[c]
C. perfringens	51.8	74.1	77.6
C. bifermentans	<0.7	1.5	14.6
C. sporogenes	<0.3	<0.3	18.8

[a] Compared with colony counts on plates treated with 250 U of unheated BLC 10 min before inoculation.

[b] BLC (preparation A) was heated in a water bath for 1 h at 65°C before use.

[c] Plates were treated with 250 U of BLC and held at 60°C for 1 h before use.

Implications for routine culturing of clostridia. The results of this study show that maximal recoveries of vegetative cells and spores of the less fastidious *Clostridium* species were obtained on plating media which had been preincubated in GasPak jars and subsequently treated with BLC before inoculation. The data also show that the presence of glucose was one of the principal factors contributing to the loss of growth-promoting ability of plating media, as had been demonstrated earlier with *C. perfringens* (5). However, this inhibitory effect was essentially absent when media were treated with BLC or preincubated in GasPak

jars before inoculation (Table 4).

Even though no attempt was made to demonstrate the presence of peroxide(s) per se in the culture medium because of the difficulties involved, the results obtained suggest that the stimulatory effect on growth of anaerobes that results from treating plates with catalase is probably due to the peroxidatic activity of the preparations (Tables 2, 6, and 9). When inhibitory concentrations of H_2O_2 were added to the culture media or when the media were subjected to UV irradiation to induce peroxide formation, the growth response of the different *Clostridium* species was found to be similar to that obtained on untreated media containing glucose and stored for prolonged periods in air. Treatment of identical plates containing inhibitory levels of H_2O_2 with catalase resulted in the restoration of most of their growth-promoting potential (Table 6). The data in Table 8 also suggest that preincubation in GasPak anaerobic jars for 18 h at 30°C is a desirable procedure prior to culturing the clostridia on ordinary plating media. Our results show that this procedure, which has been practiced extensively in some laboratories, is fairly effective in restoring the growth-promoting ability of some commonly used plating media and that treatment of media with BLC before inoculation is probably even more effective. Maximal recoveries were obtained when the two procedures were combined (Table 6). However, it should be kept in mind that catalase treatment is ineffective for culturing vegetative cells of the more sensitive anaerobes by the method we employed (Table 1). Apparently, sporulation of these species is required before they can be successfully cultured on these plating media.

Although our experiments failed to determine the precise mechanism responsible for the inhibition of the clostridia on untreated media stored in air, fermentable carbohydrate and dissolved oxygen present in the media seem to be among the most important factors contributing to loss of their growth-promoting potential. Growth-promoting ability could be restored to a large extent by omitting glucose from the medium and by preventing absorption of oxygen by the medium. Whenever these two factors were operative, preincubation in GasPak jars and treatment of the medium with catalase before inoculation were essential to good recovery on a particular medium. These results suggest that successful culturing of fastidious organisms, such as the clostridia, on blood agar that contains peroxidase can be attributed at least in part to peroxidase present in the blood. We found (unpublished data) that treating Trypticase-soy agar containing 5% sheep blood with BLC had little or no effect on colony counts with *C. perfringens*. The plates used in these experiments were obtained from a commercial supplier and were stored for about 10 days before use.

The results of this study suggest that successful quantitative culturing of *Clostridium* species on plating media requires careful handling of media to avoid loss of their growth-promoting potential. Precautions to be taken include (i) storage of the media in the dark after preparation with avoidance of exposure to strong daylight or UV irradiation, (ii) preincubation of plates in GasPak jars for several hours before inoculation, and (iii) incorporation of an active catalase or peroxidase into the medium before inoculation. Unless these precautions are taken, a poor growth response can be expected with most species.

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