

Spoilage Bacteria in Canned Foods

II. Sulfide Spoilage Bacteria in Canned Mushrooms and a Versatile Medium for the Enumeration of *Clostridium nigrificans*

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Clostridium nigrificans was found to be a spoilage organism of canned mushrooms in Taiwan. A modified beef extract tryptone iron medium, both in broth and agar form, was designed for the detection and recovery of the organisms. A procedure of simple plate counting method of *C. nigrificans* was established.

Canned mushrooms constitute one of the most important exports of Taiwan, and the production of this commodity is constantly increasing. The major spoilage problem is that due to sulfide spoilage organisms. All the organisms which have been isolated from spoiled cans are members of the spore-forming, sulfate-reducing bacteria. No suitable media for making counts of the spore-forming, sulfate-reducing bacteria are described in the literature (8). A study has been made on various types of media to find one suitable for this purpose.

MATERIALS AND METHODS

Isolation of the organisms. Cans of mushrooms taken from a batch which showed heavy sulfide spoilage were incubated at 55 C, half the cans being sampled after 7 days and the remainder after a total of 14 days. In all cases, a sample of brine was withdrawn under aseptic conditions, and a 2-ml amount was inoculated into each of four tubes of sulfite agar (12). The tubes were incubated for 72 hr at 55 C, and the black colonies were transferred into "vanilla" tubes containing the same medium for purification and isolation (9). The isolated organisms were maintained in ATCC medium code No. 42 (1), and the biological characteristics were examined by the methods described in the *Manual of Microbiological Methods* (14) and *Laboratory Methods in Microbiology* (6).

Organisms. Two strains of *C. nigrificans* were used for the comparative study of the media. The strain ATCC 7946 was obtained from the American Type Culture Collection and strain FPI 68102 was isolated from canned mushrooms opened at the Food Processing Institute. Stock cultures were maintained in ATCC medium code No. 42 (1).

Spores of these two strains were prepared as follows. The colonies of the stock cultures were inoculated into

tubes of liver broth (12), kept at 55 C for 72 hr, and then transferred into 1 liter of the same medium in long-necked flasks for cultivation. The culture tubes and flasks were stratified with liquid paraffin. After incubation for 10 days at 55 C, the cultures were filtered through a sterile Toyo no. 5 filter paper to remove liquid paraffin and sediment. After heating at 100 C for 20 min to destroy vegetative cells, the spores were separated from the medium in a centrifuge (4,000 rev/min for 15 min), suspended in sterile normal 0.85% saline, shaken with a test tube mixer to facilitate washing, and centrifuged again (4,000 rev/min for 15 min). This procedure was repeated three times. Finally, the spores were suspended in sterile normal saline to give the stock suspension.

Media. A versatile medium for determination and enumeration of *C. nigrificans* was a modification of a basal type (BETI medium). Seven liquid and 10 solid media were compared for outgrowth of *C. nigrificans* (Table 1).

Decimal dilutions of both spore suspensions of strains ATCC 7946 and FPI 68102 [stock spore concentrations: ATCC 7946, 49,000 spores/ml; FPI 68102, 24,000 spores/ml by most probable number (MPN) determination with liver broth] were prepared with sterile normal saline, and 1 ml of each dilution was inoculated into duplicate screw-capped test tubes (18 by 170 mm, outer diameter) which contained 15 ml of the test media. The liquid media were subsequently stratified with liquid paraffin, and a strip of lead acetate paper (6) was inserted with its lower end above the medium. All tubes were incubated at 55 C, and growth was observed daily for 7 days.

Plate count of *C. nigrificans*. Each spore dilution (1 ml) was transferred into each of five tubes of BETI broth and a similar amount into each of two petri dishes (bottom: 90-mm inner diameter by 20-mm depth) and subsequently mixed with 15 ml of BETI agar. After setting, the agar was stratified with approximately 70 ml of hard BETI agar. All tubes and plates were kept at 55 C for 7 days in an ordinary incubator. The MPN values were read from Sharf's table (13), depending on the number of positive tubes

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in each dilution, and the total spore counts were also determined by counting colonies on the plates. Ten different spore concentrations were studied. These were randomly selected.

TABLE 1. Media for the comparative outgrowth of *C. nigrificans*

Media no. ^a	Name
L1	Liver broth (12)
L2	Glucose liver broth (15)
L3	Baars' medium (9)
L4	Postgate's medium B (8)
L5	Cystine broth (6)
L6	Beef extract tryptone iron broth (BETI broth) ^b
L7	Canned mushroom brine ^c
S1	Sulfite agar with iron nail (12)
S2	Sulfite agar with ferric citrate (12)
S3	Iron-sulphite-agar (NORDISK) ^d
S4	0.1% lead acetate glucose agar (15)
S5	ATCC medium code No. 42 (1)
S6	Postgate's medium E (8)
S7	Modified thioglycollate agar ^e
S8	Beef extract tryptone iron agar (BETI Agar) ^b
S9	Lead acetate medium (Reed and Orr, 7)
S10	Starkey's Medium (9)

^a L, liquid media; S, solid media.

^b BETI broth and BETI agar: basal type of BETI medium contained beef extract, 3 g; tryptone, 10 g; yeast extract, 1 g; soluble starch, 1 g; dipotassium phosphate, 1.25 g; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 0.1 g; dextrose, 5 g; in 1 liter of distilled water as a liquid medium (BETI broth). The pH of the medium was adjusted to 7.0 before sterilization at 121 C for 20 min. When required in the form of a solid medium, two concentrations of agar were used: solid medium (15 g) and hard agar (20 g) in 1 liter of the above basal medium. BETI agar was used for the solid culture in tubes and on plates, and hard BETI agar was used as the agar for stratification on plate cultures.

^c Drained from commercial canned mushrooms with a pH of 6.3.

^d NORDISK, the committee for the methods of food examination, Norway. The agar contained tryptone (15 g), yeast extract (10 g), and agar (15 g) in 1 liter of distilled water. The pH was adjusted to 7.0, and the medium was sterilized for 15 min at 121 C. To 100 ml of this base medium, 1 ml of 5% ferric citrate solution, 1 ml of 5% aqueous solution of anhydrous sodium sulfite, and 1 ml of aqueous solution of potassium permanganate were added immediately before use.

^e Contained Difco Thioglycollate Medium, dehydrated, 29.8 g; sodium sulfite, 1 g; agar, 15 g; 5% ferric citrate solution, 10 ml; in 1 liter of distilled water. Sterilized in the autoclave for 15 min at 121 C; final pH, approximately 7.1.

RESULTS

Five strains were isolated from canned mushrooms, four from cans opened on the 7th day and one from a can opened on the 14th day of incubation. The isolates were rod-shaped, gram-negative, 0.4 to 0.5 by 3.0 to 6.0 μm in size, and moderately motile. They formed elliptical sub-terminal spores. The deep colonies in sulfite agar were surrounded by a blackened area of the medium. The color changed to black as a result of the fine black particles in BETI broth culture. Gelatin was not liquefied and no indole was produced. Hydrogen sulfide was produced from cystine, but acid was not produced from glucose, fructose, galactose, mannose, xylose, arabinose, rhamnose, sucrose, maltose, lactose, raffinose, starch, inulin, glycerol, mannitol, or salicin. Nitrate was not reduced to nitrite. The optimal temperature for growth was between 50 to 55 C, and the strains were obligate anaerobes.

The comparative outgrowth of *C. nigrificans* in 7 liquid and 10 solid media is shown in Tables 2 and 3.

The MPN values and the plate counts of 10 different spore concentrations of *C. nigrificans* were enumerated, and the results were shown in Table 4.

TABLE 2. Comparison of outgrowth of *Clostridium nigrificans* in liquid media^a

Media no.	Culture time	Highest dilution positive				Control tube ^b	
		Strain no. 7946		Strain no. 68102		V	L
		V ^c	L ^d	V	L		
L1	24	10 ⁻¹	10 ⁻¹	10 ⁻²	10 ⁻²	—	—
L2	24					—	—
L3	24		10 ⁻⁷		10 ⁻⁷	—	+
L4	24	10 ⁻¹	10 ⁻⁷	10 ⁻²	10 ⁻⁷	—	+
L5	24					—	—
L6	24	10 ⁻²	10 ⁻²	10 ⁻³	10 ⁻³	—	—
L7	24					—	—
L1	96	10 ⁻⁵	10 ⁻⁵	10 ⁻⁴	10 ⁻⁴	—	—
L2	96		10 ⁻¹		10 ⁻²	—	—
L3	96		10 ⁻⁷		10 ⁻⁷	—	+
L4	96	10 ⁻⁴	10 ⁻⁷	10 ⁻⁵	10 ⁻⁷	—	+
L5	96					—	—
L6	96	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵	—	—
L7	96		10 ⁻²		10 ⁻²	—	—

^a Incubated at 55 C.

^b Medium without inoculation of the organisms. No visible change was found after 96 hr of culture during 7 days of observation.

^c Visible change of the medium by growth.

^d Lead acetate paper test.

TABLE 3. Comparison of outgrowth of *Clostridium nigrificans* in solid media^a

Media no.	Culture time	Highest dilution positive	
		Strain no. 7946	Strain no. 68102
S1	24 hr		
S2	24 hr	10 ⁻¹	
S3	24 hr	10 ⁻¹	
S4	24 hr		
S5	24 hr	10 ⁻¹	
S6	24 hr	10 ⁻¹	
S7	24 hr		
S8	24 hr	10 ⁻³	10 ⁻²
S9	24 hr		
S10	24 hr	10 ⁻¹	
S1	7 days	10 ⁻³	10 ⁻¹
S2	7 days	10 ⁻³	10 ⁻¹
S3	7 days	10 ⁻³	10 ⁻²
S4	7 days	10 ⁻²	
S5	7 days	10 ⁻³	10 ⁻¹
S6	7 days	10 ⁻³	10 ⁻¹
S7	7 days	10 ⁻²	10 ⁻¹
S8	7 days	10 ⁻³	10 ⁻³
S9	7 days	10 ⁻¹	10 ⁻¹
S10	7 days	10 ⁻²	10 ⁻¹

^a Incubated at 55 C; the same spore suspension was used in the comparative study of both liquid and solid media. There was no apparent change in the control tube.

TABLE 4. Comparison of the plate counts and the most probable number values of *Clostridium nigrificans*

Strain used	Spore concentration ^a	Plate counts ^b	MPN
ATCC 7946	1	640,000	540,000
	2	24,000	33,000
	3	78	54
	4	380,000	330,000
FPI 68102	1	44,000	54,000
	2	108,000	130,000
	3	10,500	9,200
	4	5,100	4,600
	5	770,000	790,000
	6	260	330

^a Ten different spore concentrations chosen at random were used in this experiment.

^b Plates were incubated at 55 C; results are expressed as viable counts per milliliter.

C. nigrificans formed clear, whitish, disc-shaped colonies surrounded by black pigments of medium on the BETI agar plate which were stratified with sufficient volumes of hard BETI agar.

DISCUSSION

Five strains were isolated from canned mushrooms, and the experimental results showed all isolates to be *C. nigrificans* as described in *Bergey's Manual of Determinative Bacteriology* (3) except that they were gram-negative as reported by Campbell and Postgate for *Desulfotomaculum nigrificans* (5). The fact that four strains were isolated from cans incubated 7 days but only one from cans incubated 14 days might be the result of the inhibiting action of hydrogen sulfide (10) which accumulated in the contents of the can. The odor of sulfide was much more pronounced after 14 days than 7 days.

The experimental results (Tables 2 and 3) showed that the modified media, BETI broth (media no. L6), and BETI agar (media no. S8) showed a slight improvement on the recovery and on the quicker growth of *C. nigrificans* over 7 liquid and 10 solid media which were commonly used. The recovery of spores from the broth was probably 10² times higher than that of the agar tube culture.

The growth of *C. nigrificans* in liquid media could be detected by darkening of the lead acetate strips as a result of hydrogen sulfide production, and it was detectable only by this test when glucose liver broth (media no. L2) and canned mushroom brine (media no. L7) were used. It should be noted that this test can not be used as a method for detecting hydrogen sulfide production Baars' Medium (media no. L3) and Postgate's Medium B (media no. L4), since these media when incubated at 55 would show blackening of all strips, including the control, by chemically produced hydrogen sulfide.

Buften (4) examined several media and showed that none was quantitatively satisfactory for *C. nigrificans*. Postgate (11) published a procedure permitting colony counts on impure cultures and natural samples for which only MPN determinations were hitherto reliable. The experimental results, however, showed the mentioned method to be a proper procedure of simple plate culture for viable count of *C. nigrificans*.

The points of the procedure are the following.

- (i) BETI agar should be used as the plate culture medium since it improves recovery (Table 3).
- (ii) Hard BETI agar is a suitable medium as a cover for the culture medium. Two per cent agar may be adequate for the deep colonies, but it cannot detect hydrogen sulfide which is produced by surface colonies on the plates.
- (iii) The depth of the covering medium must be more than 10 mm to make the conditions sufficiently anaerobic for the growth of *C. nigrificans*.

C. nigrificans appears as whitish discus-shaped colonies surrounded by black pigments on the BETI agar plates. The viable counts obtained on agar plates and of the broth MPN values (Table 4) were not significantly different statistically (t ; $t = 0.25$, $0.8 < P < 0.9$). Even the recovery of broth cultures is probably 10^2 times higher than that of deep agar cultures (Tables 2 and 3). This procedure could also be applied to species of sporeforming, sulfate-reducing bacteria other than *C. nigrificans*.

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