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Effect of Storage Conditions on the Performance of Bismuth Sulfite Agar

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Refrigerated storage of bismuth sulfite agar plates for up to 4 days did not adversely affect growth and colonial characteristics of selected *Salmonella* strains. Incubation of inoculated plates for 48 h favored the development of more salmonellae with typical morphology. Inoculated plates of freshly poured medium incubated for 48 h gave recoveries similar to those on refrigerated plates and showed a high selectivity against *Citrobacter freundii* and *Proteus vulgaris*, organisms which mimic the colonial characteristics of *Salmonella* on this medium. The use of bismuth sulfite plates stored at room temperature for more than 2 days should be avoided.

A modification of the bismuth sulfite agar (BSA) of Wilson and Blair (11) for the isolation of *Salmonella typhi* from sewage polluted waters is commonly used for the isolation of *Salmonella* from other materials; typical colonies on this medium are black surrounded by a brownish-black halo and frequently exhibit a metallic sheen. The medium is particularly valuable for the isolation of *S. typhi* which is inhibited on brilliant green agar (7). Lack of information on the effect of aging of poured BSA plates on the selectivity and sensitivity of the medium has resulted in differing practices of storage and use. Earlier work showed that several strains of *Salmonella typhimurium* and *Salmonella enteritidis*, but not *S. typhi* or *Salmonella paratyphi* B, were greatly inhibited in freshly poured BSA and that the degree of inhibition significantly decreased with aging of the medium at refrigerator temperatures (4). Subsequent work by McCoy (8) also showed that aging in the cold was essential for the development of characteristic *Salmonella* colonies. Current *Salmonella* methodology is not consistent in its recommendations on conditions of storage and use of poured BSA plates. Reference manuals may suggest that the medium be prepared at least 48 h before use (2), used within 48 h to avoid decrease in selectivity (3), or used immediately after preparation (6). Although the U.S. Food and Drug Administration (FDA) manual (5) does not elaborate on storage conditions for BSA, plates refrigerated for 24 h have been used in FDA laboratories (1). Manufacturers (Difco, BBL) recommend that their product be used the day it is prepared. The present paper reports on the effects of temperature, light, and time of storage on the performance of BSA as a selective plating medium for the isolation of *Salmonella*.

Stock cultures of *Proteus vulgaris* (ATCC 6380), *Citrobacter freundii*, *S. typhimurium*, *S. typhi*, *Salmonella agona*, *S. enteritidis*, *Salmonella newport*, and *Salmonella infantis* obtained from the National Enteric Reference Centre, Health and Welfare Canada, were maintained on Trypticase soy agar (TSA; BBL) slants. The nonsalmonellae organisms were selected because of their ability to mimic colonial characteristics of *Salmonella* on BSA. Plates of BSA medium (Difco) were aged for up to 4 days under the following sets of conditions: room temperature (A), room temperature in the dark (B), and refrigerator (5°C ± 1°C) temperature (C). Condition (B) was studied to detect the possible formation of an inhibitor through a photodynamic interaction (10) between visible light and a photosensitizer present in the medium. Cells (1 ml) from an overnight nutrient broth culture grown at 35°C were inoculated into each of 90 ml of tetrathionate brilliant green broth (TBGB), selenite cystine broth (SCB), and nutrient broth. Test organisms were cultured in TBGB (43°C) and SCB (35°C) before their transfer onto BSA plates, to reproduce conditions that prevail in conventional *Salmonella* isolation procedures (2, 3, 5, 6). The growth of *S. typhimurium*, *S. typhi*, and of the two nonsalmonellae organisms on aged BSA was studied in two separate experiments. The growth pattern of the other *Salmonella* serotypes was determined in single experiments.

Serial dilutions (10^0 to 10^{-6}) of the TBGB and SCB broth cultures were plated on BSA aged under the selected conditions and on TSA control medium; nutrient broth cultures were plated on TSA. Plates were counted after 24 h (BSA and TSA) and 48 h (BSA) of incubation at 35°C. The data presented are the means of homologous counts which usually differed by less than 0.5 of a $\log_{10}$ unit.

Although final counts were not affected, delayed growth and formation of typical *Salmonella* colonies on freshly poured BSA plates were usually observed after 24 h with all salmonellae serotypes except *S. typhi*; definitive counts of the nontyphoid serotypes could only be obtained after 48 h of incubation. *S. typhi* was quantitatively recovered from all refrigerated BSA plates (Fig. 1); however, complete inhibition was observed on plates stored for more than 2 days at room temperature. No significant inhibition of *S. typhimurium* was observed on BSA plates aged for up to 4 days under selected storage conditions (Fig. 2). Identical growth patterns were obtained for *S. infantis* and *S. enteritidis*. The growth of *S. newport* and *S. agona*, although similar to that of *S. typhimurium*, showed significant inhibition on 4-day-old plates stored at room temperature (storage conditions [A]). Growth of *S. infantis*, *S. enteritidis*, and *S. typhimurium* on 1- and 2-day-old refrigerated plates was frequently atypical after 24 h of incubation and required an additional 24 h of incubation for the development of typical colonial characteristics. TBGB, in contrast to SCB, adversely affected the growth of most serotypes, resulting in a growth differential of approximately 2 $\log_{10}$ units with control values.

P. vulgaris, which grew only with great difficulty in TBGB, was found to be particularly sensitive to the age of poured BSA medium (Fig. 3); inhibition significantly increased with plates stored for more than 1 day at room temperature. The susceptibility of the organism to these plates may reflect its sensitivity to the increased antimicrobial activity of the oxidized (green) form of the brilliant green dye (9). The low recovery of *S. newport* and *S. agona* on 4-day-old, nonrefrigerated plates also indicates a brilliant green inhibition. Although colorless to

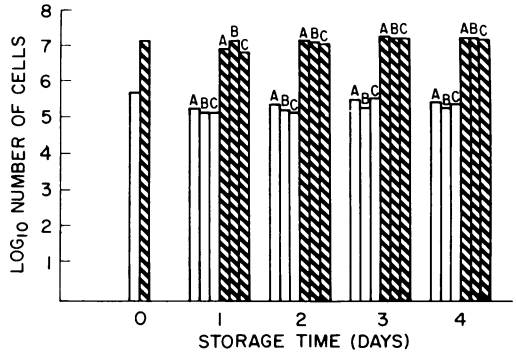

FIG. 2. *Effect of storage of BSA plates on the growth of S. typhimurium. Counts of TBGB (open bar) and SCB (hatched bar) enrichments on aged BSA after 48 h of incubation. The mean initial cell densities in TBGB and SCB as determined on TSA were 6 × 10^5 and 3 × 10^7 cells/ml, respectively. (A) Plates aged at room temperature; (B) at room temperature in the dark; (C) in the refrigerator.*

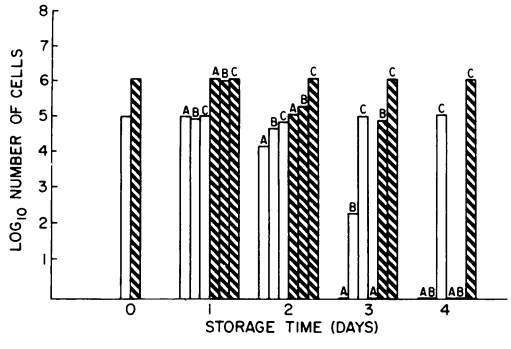

FIG. 1. *Effect of storage of BSA plates on the growth of S. typhi. Counts of TBGB (open bar) and SCB (hatched bar) enrichments on aged BSA after 48 h of incubation. The mean initial cell densities in TBGB and SCB as determined on TSA were 10^5 and 2.2 × 10^6 cells/ml, respectively. (A) Plates aged at room temperature; (B) at room temperature in the dark; (C) in the refrigerator.*

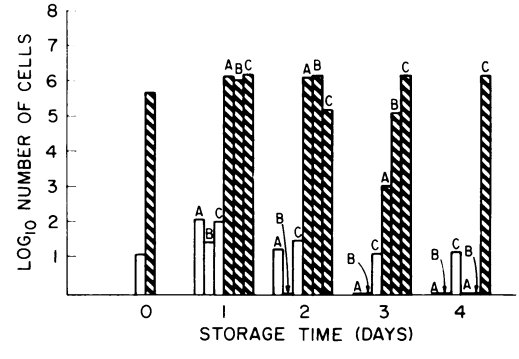

FIG. 3. *Effect of storage of BSA plates on the growth of P. vulgaris. Counts of TBGB (open bar) and SCB (hatched bar) enrichments on aged BSA after 48 h of incubation. The mean initial cell densities in TBGB and SCB as determined on TSA were 2 × 10^2 and 9 × 10^5 cells/ml, respectively. (A) Plates aged at room temperature; (B) room temperature in the dark; (C) in the refrigerator.*

light green colonies of *P. vulgaris* predominated on freshly poured (day 0) plates incubated for 48 h, the proportion of black colonies generally increased with increased aging of plates under all storage conditions.

Aging of BSA plates had limited effects on the growth of *Citrobacter freundii* (Fig. 4). Only freshly poured medium (day 0) was found to be inhibitory; counts of the SCB and TBGB on BSA were 3.0 and 1.5 $\log_{10}$ units lower respectively, than that obtained on TSA controls.

Refrigerated storage of BSA plates for up to 4 days did not adversely affect the growth and colonial characteristics of selected *Salmonella* strains, provided that inoculated plates were read after 48 h of incubation. The prolonged incubation favored the development of typical *Salmonella* characteristics and, in several enumerations, allowed for the recovery of additional colonies. Although our studies support the use of refrigerated BSA plates, results also indicate that the selectivity of these plates against *C. freundii* and *P. vulgaris* rapidly decreases with increasing storage time. Freshly poured medium performed well, allowing quantitative recovery of tested *Salmonella* strains after 48 h of incubation; the medium also exhibited high selectivity against *C. freundii* and *P. vulgaris*. Prolonged storage of BSA plates at room temperature should be avoided; although

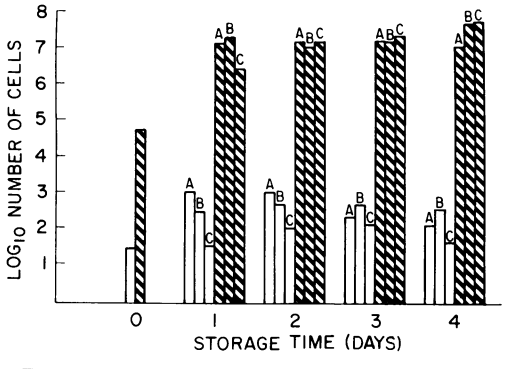

FIG. 4. *Effect of storage of BSA plates on the growth of C. freundii. Counts of TBGB (open bar) and SCB (hatched bar) enrichments on aged BSA after 48 h of incubation. The mean initial cell densities in TBGB and SCB as determined on TSA were 4 × 10² and 7 × 10⁷ cells/ml, respectively. (A) Plates aged at room temperature; (B) room temperature in the dark; (C) in the refrigerator.*

complete inhibition of *P. vulgaris* occurs on 3- and 4-day-old plates stored at room temperature (Fig. 3), the medium fails to sustain growth of *S. typhi* (Fig. 1) and inhibits other salmonellae. Our inability to demonstrate any photodynamic action indicates that storage of BSA plates in the dark is not warranted. Conclusions on the performance of aged BSA medium were based on the behavior of single strains of each test organism and do not take into account possible strain variations. Although evaluation of the performance of BSA under selected storage conditions was limited to the Difco product, similar findings would be expected with the Oxoid and BBL products which differ only qualitatively in their peptone and beef extract content.

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