ABSTRACT

Enumeration of Bacillus cereus on raw sprouts of mung beans and wheat was compared in three agars: mannitol-egg yolk-polymyxin (MYP), polymyxin pyruvate-egg yolk-mannitol-bromthymol blue, and trypsinase-soy-polymyxin blood. Ten different strains of B. cereus were used to seed the sprouts. Rates of recovery for the three media were not significantly different. However, with MYP agar, B. cereus could be differentiated more easily from other microorganisms and required fewer confirmatory tests.

Because of the increased recognition of the role of Bacillus cereus in human food poisoning, several selective plating media have been specifically developed to enumerate this organism in foods. The mannitol-egg yolk-polymyxin (MYP) agar medium described by Mossel et al. (7) has been used for more than a decade in the United States and Europe. Kim and Goepfert (4) recommended their KG agar as an improvement over MYP agar; however, KG agar has been used much less frequently than MYP agar. Holbrook and Anderson (3) developed a polymyxin-pyruvate-egg yolk-mannitol-bromthymol blue agar (PEMBA), which is similar in principle to MYP, but is considered by its originators to be superior to both MYP and KG agars. Blood agar, which is nonselective, has also been used to enumerate B. cereus, primarily in the United Kingdom (R. Gilbert, personal communication). Schieemann (8) has recommended the use of blood agar as a suitable alternative to MYP and KG agars for enumerating B. cereus in foods. Although we have used trypsinase-soy sheep blood agar, made selective by spreading a solution of polymyxin B onto the medium surface before inoculation, this medium has not been previously compared with others.

Studies comparing the different media have been limited and not generally confirmed by other published reports. As part of a study on the incidence of B. cereus in raw vegetable sprouts, we evaluated the performance of several media. MYP agar, PEMBA and trypsinase-soy-blood agar with polymyxin (TSPB) were selected for study because they appeared to be the most promising and because little information was available about the performance of the latter two. The three media were compared for enumeration, efficiency in recovering 10 different strains of B. cereus from raw vegetable sprouts, and ability to differentiate B. cereus from the other microorganisms present in the samples.

MATERIALS AND METHODS

Cultures

Ten strains of B. cereus isolated from foods associated with food poisoning were used as inocula for sprouts of mung beans and wheat. The strains were obtained as follows: B4ac, 19668 and 30020 from J. M. Goepfert, formerly at the University of Wisconsin, Madison, Wl; and F4165, F4810, F4552 and F840 from R. Gilbert, Food Hygiene Laboratory, London, England. Strains MB-31 and MB-34 were isolated in our laboratory from mung bean sprouts associated with a food poisoning outbreak. Stock cultures were maintained on nutrient agar slants stored at 4°C. Spores used in the experiments were grown on nutrient agar slants incubated at 30°C for 3 or 4 d. The growth was washed off and suspended in sterile distilled water. Spores were sedimented by centrifugation at 15,000 × g for 20 min, resuspended in sterile distilled water, washed twice by centrifugation and resuspended in 25 ml of sterile distilled water. They were stored at 4°C until needed, at which time a sufficient quantity was diluted in 500 ml of sterile distilled water to give a final concentration of 20 spores/ml.

Inoculating and sprouting procedures

A 20-g sample of mung beans or wheat seed was weighed and transferred aseptically to the sprouting bowl of a Biosta Miracle Sprouter (Miracle Equipment, Inc., Locust Valley, NY 11560) and watered with 500 ml of distilled water containing 20 B. cereus spores/ml. The water was allowed to drain into the collecting bowl of the kit and discarded. The seeds were washed twice daily with 500 ml of plain distilled water and the sprouting kits were held at room temperature on the laboratory bench until the sprouts had grown sufficiently to be harvested. The holding time was usually 2 d for wheat and 3 d for mung beans. The sprouts were collected aseptically in sterile plastic bags and either examined immediately or refrigerated overnight at 4°C and examined the following day.

Media

MYP agar was specially prepared from dehydrated MYP agar base produced for the Food and Drug Administration by Difco Laboratories (Detroit, MI). Fresh 50% egg yolk emulsion and 0.1% polymyxin B solution were prepared and used as described in the AOAC official method for enumeration of B. cereus in foods (6). PEMBA agar was prepared from the ingredients specified by Holbrook and Anderson (3). The appropriate quantities of sterile egg yolk emulsion, polymyxin B, and sodium pyruvate solution (Sigma Chemical Co., St. Louis, MO) were added before the medium was developed a polymyxin-pyruvate-egg yolk-mannitol-bromthymol blood agar medium described by Mossel et al. (7) has been used for more than a decade in the United States and Europe. Kim and Goepfert (4) recommended their KG agar as an improvement over MYP agar; however, KG agar has been used much less frequently than MYP agar. Holbrook and Anderson (3) developed a polymyxin-pyruvate-egg yolk-mannitol-bromthymol blue agar (PEMBA), which is similar in principle to MYP, but is considered by its originators to be superior to both MYP and KG agars. Blood agar, which is nonselective, has also been used to enumerate B. cereus, primarily in the United Kingdom (R. Gilbert, personal communication). Schieemann (8) has recommended the use of blood agar as a suitable alternative to MYP and KG agars for enumerating B. cereus in foods. Although we have used trypsinase-soy sheep blood agar, made selective by spreading a solution of polymyxin B onto the medium surface before inoculation, this medium has not been previously compared with others.

Studies comparing the different media have been limited and not generally confirmed by other published reports. As part of a study on the incidence of B. cereus in raw vegetable sprouts, we evaluated the performance of several media. MYP agar, PEMBA and trypsinase-soy-blood agar with polymyxin (TSPB) were selected for study because they appeared to be the most promising and because little information was available about the performance of the latter two. The three media were compared for enumeration, efficiency in recovering 10 different strains of B. cereus from raw vegetable sprouts, and ability to differentiate B. cereus from the other microorganisms present in the samples.
plated. Twenty ml of complete medium was poured into 100 x 15 mm petri plates, and the plates were allowed to dry for 48 h at room temperature before use.

Trypticase-soy sheep blood agar plates containing 5% defibrinated sheep blood were purchased from England Laboratories, Beltsville, MD. The plates were stored at 4°C for a maximum of 7 d before use. Just before inoculation, 0.1 ml of sterile 0.2% polymyxin B solution was spread evenly onto the surface of each plate, and the plates were allowed to dry for 15 min at room temperature.

**Bacteriological examination**

A 50-g sample of sprouts was examined as described in the AOAC official method for enumeration of *B. cereus* in foods (6) except that replicate portions of each sample dilution were plated in duplicate on PEMBA and TSPB agars in addition to MYP agar as specified in the method. The plates were incubated for 20 to 24 h at 30°C, the presumptive *B. cereus* colonies were counted and 5 colonies were transferred from each medium to nutrient agar slants for confirmation as *B. cereus*. The isolates were identified by the method of Harmon (2).

**Statistical analysis**

The study was performed in a split plot design by substrate. The data were analyzed for the effects of media, strains and sample dilution. The data obtained with mung bean sprouts and wheat sprouts were analyzed separately. Counts were expressed as logarithms for the analysis of variance. Calculations for the three-way design with interactions have been described by Kirk (5). Duncan's multiple range test (7) was used to determine which means differed.

**RESULTS AND DISCUSSION**

The results obtained with mung bean sprouts are presented in Table 1; those obtained with wheat sprouts are shown in Table 2. The *B. cereus* counts were quite similar on all three media. Although counts on TSPB medium were usually slightly lower than those on MYP and PEMBA, the differences were small and not statistically significant (p>0.05) at the population levels present in the samples. A summary of the statistical analysis of the plate count data is presented in Table 3. Because of the disparity between the counts on mung bean sprouts and those on wheat sprouts for the same strains of *B. cereus*, the data obtained with the two types of sprouts were analyzed separately. No significant differences were noted among the means for the media or the sample dilutions; however, the means for the strains at the same dilution varied significantly (p<0.01). The interaction involving media and strains at the same dilution was also significant with both substrates (p<0.05).

Because all three media gave essentially the same counts for the same set of circumstances, the choice of a medium for enumeration of *B. cereus* in foods such as vegetable sprouts depends on factors other than quantitative recovery. The three media, therefore, were also evaluated for their ability to suppress growth of organisms other than *B. cereus* and for differentiation of *B. cereus* from other bacteria which grew.

None of the media was more proficient than the others in suppressing the growth of contaminants, which often developed in large numbers on all three media. Although this usually did not interfere with the counting of presumptive *B. cereus* colonies, as noted by Schiemann (8), it complicated the selection of colonies for confirmation tests by making it necessary to subculture and streak plates to obtain pure cultures. However, *B. cereus* colonies developed rapidly on all three media and could usually be distinguished from other bacteria without difficulty.

All of the *B. cereus* strains produced typical presumptive (lecithinase-positive/mannitol-negative) colonies on MYP agar. Lecithinase-positive colonies were also produced by all strains on PEMBA agar; however, the alkaline reaction, which indicates that mannitol was not fermented, was frequently lacking on PEMBA when the colonies were counted. Thus, before further identification tests were made, many isolates from PEMBA had to be tested to be sure they did not ferment mannitol. We considered this to be a definite disadvantage.
Although TSPB agar does not contain mannitol, the strong hemolytic action of \textit{B. cereus} could be relied upon for differentiating its colonies from those of other bacteria. We found hemolysis to be as constant a character of \textit{B. cereus} as the lecithinase and mannitol reactions and equally useful in distinguishing \textit{B. cereus} from other bacteria.

In addition, the tendency of \textit{B. cereus} to spread on PEMBA and coalesce with adjacent colonies made counting difficult when >30 colonies were present. Although all \textit{B. cereus} strains sporulated more abundantly on PEMBA than on the other two media, this was not an advantage because all \textit{B. cereus} isolates sporulated abundantly on nutrient agar slants. Abundant sporulation would be useful only if the staining method of Holbrook and Anderson (3) were used to confirm \textit{B. cereus} isolates. This method, however, is not sufficient for differentiating \textit{B. cereus} from other species in the genus \textit{Bacillus} (2).

It was concluded that MYP is slightly superior to the other two media because on it, colonies of \textit{B. cereus} are more easily differentiated from those of other species. On MYP there is no need for a separate mannitol fermentation test which, although not essential for confirming isolates, is helpful in differentiating \textit{B. cereus} colonies. The ability of \textit{B. cereus} to ferment mannitol is a characteristic that differentiates this organism from many other \textit{Bacillus} species (2,3,6,7).

Although we preferred MYP medium for these reasons, our results indicate that the other media would be acceptable alternatives if a mannitol fermentation test were included as part of the confirmatory procedure.

REFERENCES