BIOTYPING AND SEROTYPING OF *Bacillus cereus* ISOLATED FROM FRIED RICE AND CHAWMIN

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(Certificate of the Major Advisor
and endorsement of the Head of the
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CERTIFICATE

This is to certify that the thesis entitled "BIOTYPING AND SEROTYPING OF *Bacillus cereus* ISOLATED FROM FRIED RICE AND CHAWMIN" submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy of Veterinary Science (Veterinary Public Health & Epidemiology) of the Faculty of Post-Graduate Studies, Birsa Agricultural University, Ranchi (Bihar) is the record of bonafide research carried out by Dr. Rajendra Yadava under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that such help or information received during the course of this investigation and preparation of the thesis have been duly acknowledged.

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We, the undersigned, members of the Advisory Committee of Dr. Rajendra Yadava a candidate for the Degree of Doctor of Philosophy of Veterinary Science with major in Veterinary Public Health & Epidemiology have gone through the manuscript of the thesis and agree that the thesis entitled "BIOTYPING AND SEROTYPING OF *Bacillus cereus* ISOLATED FROM FRIED RICE AND CHAWMIN" may be submitted by Dr. Rajendra Yadava in partial fulfilment of the requirements of the degree.

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This is to certify that the thesis entitled "BIOTYPING AND SEROTYPING OF *Bacillus cereus* ISOLATED FROM FRIED RICE AND CHAMMIN" submitted by Dr. Rajendra Yadava in partial fulfilment of the requirements for the Degree of Doctor of Philosophy of Veterinary Science (Veterinary Public Health & Epidemiology) of the Faculty of Post-Graduate Studies, Birsa Agricultural University, Ranchi, Bihar was examined and approved on 12th October, 1993
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INTRODUCTION

Bacillus cereus food poisoning was documented in 1950 by Hauge. Since then a large number of incidents have been reported in many countries. B. cereus produces two clinical forms of food poisoning. The diarrhoeal type, caused by diarrhoeagenic toxin, is characterised by diarrhoea commencing 8 to 16 hours after ingestion of a contaminated food, seldom accompanied by vomiting or fever. This food poisoning resembles the one caused by Cl. perfringens. The other one is emetic type, which is characterised by nausea and vomiting commencing 1 to 5 hours after ingestion of a contaminated food. The syndrome resembles that of staphylococcal food poisoning. The enterotoxin is designated as emetic type.

A wide variety of foods have been implicated including meat and vegetable soups, cooked meat and poultry, raw and cooked vegetables, various dessert dishes and occasionally such foods as fish, pasta, milk and ice-cream (Gilbert, 1979). Rice dishes are almost always associated with outbreaks of emetic type. Other foods, such as poultry and vegetable dishes, milk and milk products, infant foods, omelette, pancake and vanilla sauce have also been implicated.

Boiled and fried rice prepared in restaurants (many of which serve Chinese style foods) have been implicated in outbreaks of emetic type of B. cereus food poisoning in the United Kingdom, Canada, the Netherland and United States. Large number (10^5 to 10^9/g) of B. cereus have been isolated from remnants of rice in outbreaks.

Despite under reporting in India two events of B. cereus food poisoning/ gastroenteritis were reported (Kulshreshtha, 1978; Lakhani, 1979). Deva (1989) observed 50.0 to 65.0 per cent of Salami and Trekker to be positive for Bacillus spp. Some isolates were later identified as B. cereus (Singh, 1990), Mondal (1990) reported 10.0 to 15.0 per cent of Frankfurter and its ingredients positive for B. cereus.

Chinese foods and fast food restaurants serving these are increasingly becoming popular. Chances of contamination of these foods with B. cereus and resulting food poisoning have thus increased. It is high time that studies on incidence and level of contamination of such foods with B. cereus in India be worked out, as no such information is available.

Jha (1992) used 18 sugars to study fermentative ability of B. cereus. The isolates under tests were found variable with respect to fermentation of xylose, salicin and cellobiose. Fermentation of xylose was considered important for differentiation of Indian strains from British ones. A biotyping system was suggested on the basis of fermentation of these three sugars.

Serotyping of B. cereus has been described. But the typability is poor. Gilbert and Taylor (1976) reported that the majority (38 of 66 i.e. 57.6%) of B. cereus isolated from uncooked rice remained untyped by their 18 sera used for serotyping. While Gilbert and Parry (1977) reported that atleast 40.0 per cent of cultures were not typable by British serotyping scheme. Similar evidence was presented by Ghosh (1978) who found

that 52.0 per cent isolates of B. cereus from faecal specimens remained untyped. Further, all of 26 isolates of B. cereus sent from this Laboratory to PHLS, Colindale were untypable. In the identification of Japanese isolates a battery of 12 antisera was added to the British serotyping system (Terrayama et al., 1978).

A serotyping system using battery of antisera raised against Indian isolates was desired. Jha (1992) made an attempt and raised battery of 17 sera. Results were encouraging though he experienced many cross reactions. The strains used for raising antisera originated mainly from pork products and, therefore, cross reaction was expected.

The present study was undertaken with following objectives :

1. Examination of foods from Fast Food Restaurants to elucidate contamination with B. cereus.
2. Estimation of levels of contamination of Chinese foods with B. cereus.
3. Biochemical characterisation of isolates and their biotyping.
4. Serological identification of isolates.

REVIEW OF LITERATURE

The genus Bacillus was created by Ferdinand Cohn in 1872. The species established was B. subtilis. Frankland and Frankland (1887) described the species of B. cereus. The Bacillus spp. was first classified by Smith et al. (1952). They also proposed a taxonomic scheme for the separation and identification of the aerobic spore bearing bacilli that forms the basis of current procedures till now.

The first well documented report on food poisoning due to B. cereus was published in 1950 (Hauge, 1950). The organisms implicated in food borne illness were incompletely described as 'anthracoid' Bacillus or as a member of the 'subtilis mesentericus' group or as 'pseudo anthrax like'. A number of reports on non-gastrointestinal episodes were published during the same period. After the publication of first well-documented report on food poisoning caused by B. cereus (Hauge, 1955), B. cereus has been established as food poisoning organism and as a direct or opportunistic pathogen in non-gastrointestinal infections also (Goepfert et al., 1972; Gilbert, 1979; Turnbull et al., 1979; Gilbert et al., 1981; Jones and Turnbull, 1982). B. cereus caused a number of disease such as bacteraemia and septicaemia (Curtis et al., 1967; Crowley, 1970; Barnham and Taylor, 1977), endocarditis (Craig et al., 1974; Block et al., 1981), pneumonia and pleurisy (Stopler et al., 1964), gas gangrene like infection (Grosschel et al., 1976; Turnbull et al., 1977), cerebral necrosis (Turnbull et al., 1977), osteomyelitis (Seiny et al., 1977), urinary tract infection (Melles et al., 1969) and meningitis (Leffert et al., 1970; Raphael and Donaghue, 1976).

B. cereus also causes a number of infections in domestic animals. These are gangrenous mastitis in cows (Jasper et al., 1972; Wohlgemuth et al., 1972), abortions in cattle (Wohlgemuth et al., 1972), severe and fatal cases of bovine mastitis (Anonymous, 1977). Cases of acute mastitis have also been reported in dairy cows by Fabbi et al., (1989). This organism was isolated from mastitic milk and the udder tissue of the affected cows.

A number of reports were reviewed by Gilbert et al. (1981) involving B. subtilis, B. licheniformis and B. brevis as the food poisoning agents.

B. subtilis and B. licheniformis can cause 'opportunistic' infection in clinically compromised hosts combining with B. marcerans, B. circulans, B. pumilus and B. sphaericus (Farrar, 1963; Pearson, 1970; Tuazon et al., 1979; Gilbert et al., 1981).

Characters of genus Bacillus :

The genus Bacillus can broadly be defined as comprising Gram positive or variable rod shaped bacteria capable of forming refractile endospore in an aerobic environment. Except for few strains, the bacteria of this genus produce catalase (Gordon et al., 1973). The endospore are highly resistant to heat, dehydration and other destructive factors and all members of genus Bacillus exist as saprophytes in soil, water, air and on vegetation. Few species have distinct habitats (Gibson and Gordon, 1974).

Characters of *Bacillus cereus* :

B. cereus is a Gram positive, motile facultative anaerobic, endospore forming bacilli. Vegetative cell dimensions are typically 1.0 to 1.2 μm by 3.0 to 5.0 μm and the ellipsoidal spores are formed in central or paracentral position without swelling the sporangium. The organisms grow in a temperature range of 10°C to 48°C with optimum growth occurring between 28°C and 35°C. Widely variable characters of *B. cereus* have been described by Gibson and Gordon (1974), Cowan (1974) and Norris *et al.* (1981). Non motile strain of *B. cereus* were also reported, hence the manuals on determinative bacteriology include motile/ non-motile both.

On the basis of the large number of isolates of *B. cereus* Gilbert *et al.* (1981) suggested that *B. cereus* is motile, forms characteristic colony on 5.0 per cent horse blood agar, is lecithinovitelline positive and ferments glucose but not arabinose, xylose and mannitol in ammonium salt sugar bases. For suspected *B. cereus* colonies giving variable or doubtful reactions and colonies of other *Bacillus* spp., they suggested a set of 15 tests for screening.

Distribution of *Bacillus cereus* in nature :

Spore and vegetative forms of *B. cereus* are commonly found in varied environments such as soil, sediments, dust, natural waters vegetation and many kinds of foods specially milk and dairy products, herbs, spices, cereals and cereal products,

meat and poultry (Goepfert et al., 1972; Norris et al., 1981). According to Autai (1988) unprocessed spices (aligator peppia, red pepper, black pepper, thyme and curry powder) were highly contaminated and B. cereus was isolated in high numbers in majority of 230 samples he examined. Ueda and Kuawara (1990) found that all the 111 soil samples from vegetable farm land were positive for B. cereus ranging between 10^3 to 10^7/g. The isolates were also diarrhoeal toxin producers. Ueda et al. (1991) examined 185 soil, 28 vegetable samples from 88 fields and 205 vegetable and shredded vegetable samples from retail shops. 91.0 per cent of soil and 55.0 per cent of vegetable samples were positive for B. cereus in the range of 10^2 to 10^7 cfu/g and 10^2 to 10^5 cfu/g respectively. 20.0 per cent of vegetable samples from market contained B. cereus 10^2 to 10^5 cfu/g. Due to its wide-spread natural distribution B. cereus is inevitably ingested from time to time and contributes as transitory intestinal flora. It has been isolated from the faeces of healthy children. It varied from 14.0 per cent in United Kingdom (Ghosh, 1978), 15.0 per cent in Japan (Itoh et al., 1982), 24.0 to 43.0 per cent in South Africa (Turnbull and Kramer, 1985).

Bacillus cereus food poisoning :

Outbreaks of food poisoning have been reported throughout Europe, and also in Australia, Canada and the USA (Hauge, 1950; 1955; Goepfert et al., 1972; Gilbert, 1979). Abdominal pain, profuse diarrhoea, tenesmus, nausea appearing 10 to 12 hours

after ingestion of contaminated food and recovery in 12 to 24 hours were characteristics symptoms.

In 1974 Mortimer and McCann reported several outbreaks of a different clinical form which followed the ingestion of fried rice. Each episode was characterised by an acute attack of nausea and vomiting with an incubation period of 1 to 5 hours. In some cases diarrhoea was also observed. The clinical course lasted for less than 24 hours. More than 120 such episodes have since been reported in United Kingdom and in the vast majority fried or boiled rice usually from Chinese restaurant or 'take-aways' was the only food common to those affected. Similar outbreak have been reported from other parts of the world (Gilbert, 1979).

B. cereus thus produces two distinct types of food poisoning, an emetic type, similar in nature to staphylococcal food poisoning most frequently also associated with cooked rice and a 'diarrhoeal' type, closely resembling Cl. perfringens food poisoning and in which a number of varied foods have been implicated. Kramer et al. (1982) reported that Rhesus monkey feeding test demonstrated both emetic and diarrhoeal types of enterotoxins of B. cereus. However, this test is costly and cannot be used on routine basis and was also least dependable (Thompson et al., 1984).

Midura et al. (1970) reported first well documented food poisoning outbreaks of B. cereus affecting 15 members of a fraternity at the University of California, Berkeley. The

students developed an illness characterised by diarrhoea, abdominal pain, nausea with little vomiting and little or no fever. The average incubation period in this outbreak was 10 hours. Laboratory examination of the suspected food revealed 70×10^6 B. cereus per gram of meat loaf but the more common agents usually incriminated in bacterial food poisoning were not observed. The meat loaf was the most likely vehicle for the organism in each food poisoning outbreak.

Portnoy et al. (1976) reported an outbreak of B. cereus food poisoning in Houston, Texas, resulting from contaminated vegetable sprouts. Victims developed symptoms after an incubation period of 6 to 15 hours and illness was characterised as nausea and vomiting followed by abdominal cramp and diarrhoea in three cases on bacteriological investigation. B. cereus was found in excess of 10^7/g in vegetable sprouts. B. cereus isolated from the incriminated sprouts exhibited enterotoxigenic activity when tested by ligated rabbit ileal loop technique, dermal reaction in guinea pig, vascular permeability reaction in rabbits.

In Hungary, many outbreaks have been reported. According to Ormay and Novotny (1970) B. cereus was the third most common cause of bacterial food poisoning in between 1960 and 1968 being responsible for 8.4 per cent of reported outbreaks and 14.8 per cent of reported cases.

Isolation of *Bacillus cereus* :

Of the food and food ingredients examined by Nygren (1962) in Sweden 47.8 per cent were contaminated with *B. cereus*.

The organism is of particular concern in raw and pasteurised milk. It causes spoilage which is known as 'bitty' or 'broken' cream (Stone, 1952a ; Stone and Rowland, 1952b).

Ionescu *et al.* (1966) examined raw and pasteurised bottle milk. They reported *B. cereus* counts ranging from 10^1 to 10^4/ml in 12.4 per cent of fresh raw milk, 86.7 per cent of bottled pasteurised milk and 100.0 per cent in samples directly taken from pasteurizer. Ahmed *et al.* (1983) reported the isolation rate of *B. cereus* as 9.0 per cent from raw milk, 45.0 per cent from pasteurised milk, 14.0 per cent from cheddar cheese and 48.0 per cent from ice-cream at levels upto 100/ml, 1000/ml, 200/gm and 3800/gm, respectively.

Nagarajan *et al.* (1990) reported that a psychrotrophic *B. cereus* strain, isolated from milk stored at 4°C, inoculated in fresh sterile milk was able to survive pasteurization at 70°C for 15 minutes or 100°C for 10 minutes mainly as spores. Harmon and Kautter (1991) examined 18 rehydrated non fat dry milk and found them positive for *B. cereus* with counts ranging from 0.15 to 5.0 $\times 10^6$/g after 20 hours and 5.0 to 49 $\times 10^6$/g after 24 hours incubation at 26°C. *B. cereus* counts in the powdered milk were low, average being 0.29/g. Sallam *et al.* (1991) examined 200 samples of milk and different dairy products

for incidence of B. cereus. B. cereus was detected in 35.0, 15.0, 37.5, 27.5 and 12.5 per cent of raw milk, pasteurized milk, ice-cream, Domiati cheese and Kareish cheese samples, respectively. Mean B. cereus counts were 2.190×10^3/ml, 1.752×10^2/ml, 2.417×10^3/g, 1.337×10^2/g and 39.0 ± 14.31/g, respectively.

Grewal and Tiwari (1990) reported aerobic plate count in samples of rasmalai ranging from 10^2 to 10^6 cfu/g. B. cereus was isolated in 32.0 per cent of the samples examined.

Glenn et al. (1988) examined 30 samples of fried rice, 10 were positive and counts in 5 of these were between 10^4 to 10^9/g. Kamat et al. (1989) examined 124 different foods from the local market and found 28.5 per cent of rice and rice products to be positive for B. cereus.

Harmon and Kautter (1991) surveyed the incidence of B. cereus in different types of foods and reported that 88 of 106 (83.01 %) ready to serve moist foods were positive for B. cereus at levels ranging from 0.25 to 8.5×10^6/g after 20 hours of incubation and from 0.1 to 58×10^6/g after 24 hours.

Todd (1992) studied 10 year of food borne disease data from 1975 to 1984 in Canada. Micro-organisms particularly Salmonella, S. aureus, Cl. perfringens and B. cereus were main etiological agents. Most of the problems associated with food borne illness occurred at food service establishments, but the impact of mishandling at houses and food processing establishments,

was also great.

B. cereus food poisoning in India was first reported by Kulshreshtha in 1978 among children. B. cereus was isolated from stool, vomitus and milk powder. Another, outbreak was reported by Lakhani (1979) in which 500 persons were affected in a religious function near Poona. B. cereus was isolated from rice. The count of B. cereus in rice was 2.7 x 10^7/g. The predominant symptoms observed were nausea and vomition.

Sherikar et al. (1979) examined 95 samples of different ready to cook pork products such as cocktail sausages, Oxford sausages, porkis, ham, bacon and kabab for microbial load. They found 38.94 per cent samples positive for B. cereus.

Khot and Sherikar (1984) found 4.2 per cent and 11.42 per cent samples of uncooked cocktail sausage obtained from market of Bombay, positive for staphylococci and B. cereus, respectively.

Kulshreshtha et al. (1984) examined the cases of acute gastroenteritis during five year period (1978 - 82). They isolated large number of B. cereus from stool and vomitus. They found 10^3 to 10^7/gm B. cereus counts in milk powder.

Choudhary et al. (1986) reported incidence of B. cereus upto the extent of 4.76 per cent in 105 diarrhoeal stool samples.

Khot and Sherikar (1986) examined microbial flora of 112 samples of patties (49 mutton, 37 chicken and 26 beef patties) and reported 57.15 per cent of mutton patties,

66.76 per cent of chicken patties and 92.31 per cent of beef patties positive for B. cereus.

Varadaraj et al. (1992) isolated B. brevis, B. cereus, B. circulans, B. coagulans, B. laterosporus, B. licheniformis, B. pumilus, B. stearothermophilus and B. subtilis from Indian snack and lunch foods. They observed higher count of 4.2 $\log_{10}$ (cfu/g) and a lower count of 2.6 $\log_{10}$ (cfu/g) in bisibele bhath and uppuma, respectively. It was also observed that the different Bacillus species occurring as post processing contaminants in plain cooked rice reached cell populations which were sufficient to cause health hazards.

Yusuf et al. (1992) assayed flours and meals (maize, sorghum, millet, yams, cassara and gari) for B. cereus contamination. A total of 300 samples examined 28.0 per cent contained B. cereus at 1×10^3/g, 64.7 per cent at 1×10^4/g and 7.3 per cent at 1×10^5/g. Of the 72 isolates tested for enterotoxin production, 45.8 per cent were positive.

Tariwate et al. (1993) isolated different species of Bacillus viz., B. cereus, B. subtilis, B. megaterium, B. circulans, B. coagulans and Bacillus species from different points in a slaughter house.

Attention to Indian isolates being different was drawn when the PHLS, London studied 3 isolates of Deva (1989) from Salani and Trekker. These isolates were, serologically untypable, xylose positive and resembled cluster of emetic type enterotoxin.

Singh (1990) studied the isolates in detail and found that they produced diarrhoeal type enterotoxin.

The observations of Deva (1989) and Singh (1990) were confirmed by Mondal (1990). He isolated 22 strains of B. cereus from frankfurter sausage, wheat flour and spices. All the isolates were xylose positive, Y phase resistant and serologically non typable with British serotyping system at PHLS, Colindale and were VPR positive. All xylose positive isolates were salicin negative or vice-versa.

Jha (1992) reported that 42 strains of B. cereus were distributed into seven different biotypes. For precise identification of an isolate of B. cereus both biotyping and serotyping was recommended.

Singh et al. (1992) attempted to elucidate the possible mechanism of B. cereus pathogenesis. They suggested that the toxin did not affect the (Na^+ - K^+) ATPase, a membrane bound enzyme of intestinal epithelial cells unlike in case of Cl. perfringens type A enteropathy.

Properties of partially purified toxins :

Gilbert and Kramer (1984) reported the properties of partially purified toxins as follows :

Diarrhoeal enterotoxin :

It is protein in nature. The molecular weight varies

from 50,000 to 57,000, isoelectric point from 4.9 to 5.3. It is unstable at pH $\angle$4 and $\rightarrow$ 11. It is destroyed at 55°C for 20 minutes. It is sensitive to pronase and trypsin but resistant to EDTA. B-glucuronidase and alkylation. The optimum temperature for production is 32°C to 37°C and is produced during late exponential phase of growth. It is lethal to mice and can be detected by RLIL, VP, tissue culture assay and immunogel diffusion tests.

Emetic toxins :

It is a small peptide with molecular weight $\angle$5000. It is stable at 126°C for 90 minutes and between pH 2 to 11. It is resistant to pepsin and trypsin. The optimum temperature for toxin production is 25°C to 30°C and is produced during stationary phase. It is non-antigenic in nature. Laboratory detections may be done by monkey feeding test.

Enterotoxin :

Gilbert and Kramer (1986) reported that toxin was unstable during its storage at 4°C or 25°C and was inactivated at 56°C for 5 minutes but was stable at 45°C for 30 minutes (Spira and Goepfert, 1975). They further reported that the toxin was unstable under a wide variety of conditions, ionic environment, pH and temperature.

Singh (1990) observed that VPR activity of the toxin was most labile and sensitive to heat (45°C for 30 minutes), enzymes (trypsin and pepsin) and extremes of pH ($\angle$6.0 and

7 8.0) but resistant to EDTA. The hemolysin was destroyed by heat (45°C for 10 minutes) and was sensitive to trypsin and pepsin and pH 10.0 and 11.0 but was resistant to acidic pH. The phospholipase activity of the toxin was most resistant. It was stable to heat (100°C for 15 minutes), enzymes (trypsin and pepsin) and extremes of pH (2.0 to 11.0).

Spira and Goepfert (1975) demonstrated that the toxin responsible for diarrhoeal sympton is a true enterotoxin, capable of eliciting fluid accumulation in the rabbit ileal loop, increasing vascular permeability in the rabbit skin test and lethal to mice upon intravenous injection.

Melling et al. (1976) using oral challenge of rhesus monkey, determined that the strain of B. cereus isolated from vomiting type episodes and grown in rice slurry culture elicit an acute emetic response without accompanying diarrhoea, whereas isolates from diarrhoeal syndrome outbreak in the same model elicit a predominantly diarrhoeal response.

The vascular permeability reaction (VPR), shown also in the context of cholera toxin to be related to fluid accumulation factor in LRIL (Craig, 1971) was found to be applicable to studies of the B. cereus toxin (Glatz and Goepfert, 1973) and a correlation between these activities and induction of diarrhoea in monkeys was also reported (Goepfert, 1974).

Turnbull et al. (1979) reported VPR activity in 36 isolates of B. cereus from foods. They considered VPR as more dependable a test than LRIL in which the dilatation indices of any one strain varied widely.

Kramer et al. (1982) reported that rhesus monkey feeding with B. cereus toxins (emetic and diarrhoeal) had been effective. Feeding tests in rhesus monkeys have shown that the cultural conditions necessary for obtaining a reproducible response in animals are hard to establish. Goepfert (1978) reported that whole cell culture of B. cereus strain B-4ac, isolated from a diarrhoeal type outbreak, elicited a diarrhoeal response in rhesus monkeys. Similar tests using broth cultures from vomiting type outbreaks did not induce vomiting, the response was either diarrhoea or no effect (Goepfert, 1974). However, feeding tests in rhesus monkey using slurries of rice cultures containing large numbers (Ca $1x10^{10}$ cells) of B. cereus have been successfully used both to fulfill Koch's postulates for a number of vomiting type outbreak isolates and to characterize the factor responsible (Melling and Capel, 1978; Melling et al., 1979; Turnbull et al., 1979b). However, this test is costly and cannot be used on routine basis and was also least dependable (Thompson et al., 1984).

Serotyping scheme of *Bacillus cereus* :

The antigens of different *Bacillus* species were studied by Norris and Wolf (1961) who demonstrated that spore antigen possessed the highest species specificity while the flagellar antigen showed highest degree of strain specificity.

Studies with spore antigen gave better results. Norris and Wolf (1961) reported that a heat resistant, hot formamide extractable precipitinogen that was species specific was present in spores of *B. cereus*. However Kim and Goepfert (1971) were unable to confirm the species specificity of this antigen.

Kim and Goepfert (1972) used the fluorescent antibody technique and the globulin fraction of the serum prepared against autoclaved spores of *B. cereus* strain *T*. Fluorescence at the surface of spores was seen in 58 out of 59 strains of *B. cereus* and with the other strains tested such as *B. anthracis*, *B. mycoides* and *B. thurigiensis*.

On the basis of somatic antigen used for serological identification, only little success has been achieved with somatic antigen. Although no definite typing scheme has been developed, spore antigens have given better results (Gilbert *et al*., 1981).

A serological typing scheme based on flagellar antigen was developed by Taylor and Gilbert (1975) and Gilbert and Parry (1977), of Food Hygiene Laboratory, London, for the investigation

of food poisoning outbreaks. In this scheme are 28 agglutinating antisera against the H antigen of prototype strains of B. cereus isolated from foods, clinical specimens and food poisoning outbreaks in Great Britain, the Netherlands, Canada and the USA and one from a neonatal abscess.

A battery of 12 antisera was added to the British serotyping system for the study of incidence of B. cereus in various foods in Japan by Terrayama et al. (1978).

Jha (1992) raised a battery of 17 antisera against Indian isolates. Results were encouraging though he experienced many cross reactions. The strains used for raising antisera mainly originated from pork products and therefore, cross reaction was expected.

The British serotypes 2, 6, 8, 9 and 10 were isolated from the food poisoning cases in USA, Canada and continental Europe. These serotypes were characterized by a longer incubation period. The emetic illness associated with rice was reported in Great Britain and Australia. The serotypes involved were 1, 3 or 5 (Taylor and Gilbert, 1975).

Gilbert and Parry (1977) reported distribution of 400 isolates of B. cereus from various routine samples of food not associated with an outbreak of food poisoning. Twenty one of the 23 recognised serotypes were isolated, although for all the foods, atleast 40.0 per cent of the culture were not typable. Type 1 was the most common serotype isolated. For boiled and fried rice, 23.0 per cent of the cultures were type 1

compared to only 3.0 per cent for uncooked rice. In contrast, 15.0 per cent of the cultures from uncooked rice were type 17 compared with only 1.0 per cent for cooked rice. Several food samples, particularly uncooked rice, yielded multiple serotypes.

Ghosh (1978) found that 15 of the 23 serotypes of B. cereus in the serotyping scheme were represented among 100 strains isolated from faecal specimens of healthy adults, 52 of the 100 isolates (52.0 %) were not typable.

Gilbert (1979) reported that in 50 of the 86 outbreaks associated with vomiting type syndrome, foods, clinical specimens or both yielded serotype 1 only. In 10 episodes type 1 strain was isolated together with other serotypes also. The other serotypes involved in the vomiting type outbreaks were types 3, 4, 5, 8, 12 and 19. Two of the diarrhoeal type outbreaks yielded serotype 1 only. Types 2, 6, 8, 9, 10 and a mixture of type 12 and untypable strains appeared to be responsible for one outbreak each. Types 1, 8 and 12 have been implicated in both the vomiting and diarrhoeal type outbreak. It was also concluded that outbreaks of food poisoning involving B. cereus together with other organisms might occur and serotyping may be useful in investigation (Gilbert, 1979).

Shinagawa et al. (1980) concluded that most of the emetic type outbreaks were due to serotype 1 only. The involvement of serotype 1 may be explained due to its greater heat resistance in aqueous suspension and rice during cooking(Parry and Gilbert, 1980).

DeBuno *et al.* (1988) applied the plasmid analysis alongwith serotyping and enterotoxin analysis of *B. cereus* in an outbreak associated with consumption of beef stew. They observed that all isolates had identical plasmid profile which differed from those *B. cereus* strains which were selected randomly from other outbreaks. Thus, they concluded that the plasmid profile may prove an useful tool in the investigation of *B. cereus* food poisoning.

MATERIALS & METHODS

Collection of samples for bacteriological examination :

The following samples were collected asceptically from a Fast Food Centre, Ranchi and brought to the laboratory. The details of the samples examined is presented in Table 1.

Table 1. Summary of the samples examined.

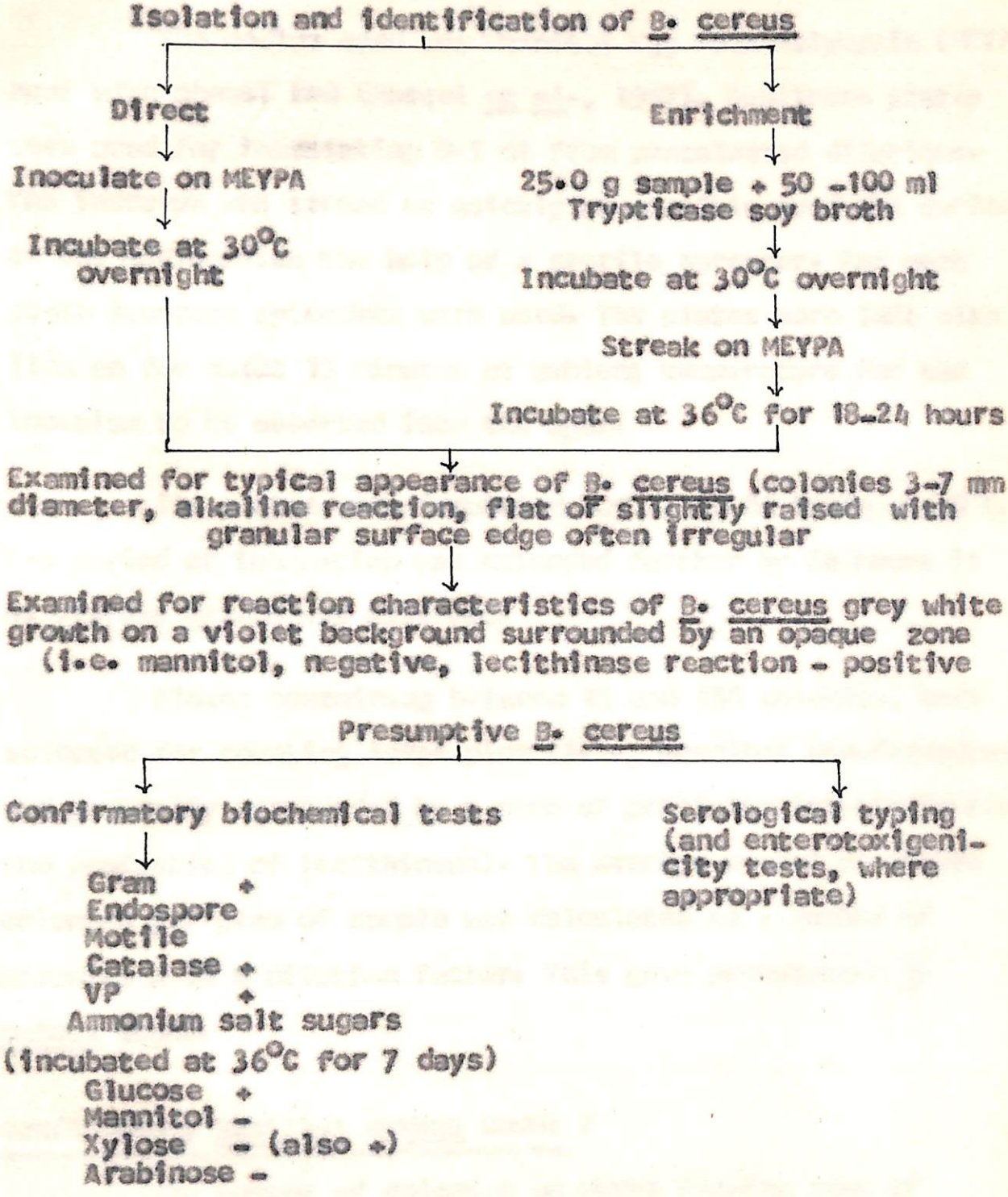
Sl. No.	Name of the samples	No. of samples examined	Sources
1	Fried rice	100	Fast Food Centre, Ranchi.
2	Chawmin	100	Fast Food Centre, Ranchi.
3	Skimmed milk powder (Indana)	26	Mice Breeding Unit, Animal Breeding & Genetics, Ranchi Veterinary College, Ranchi-7.

The samples were processed immediately for bacteriological examination.

Preparation of inocula :

Each sample weighing 7.0 gm was taken in sterile mortar and pestle and minced properly. The minced sample was transferred to a conical flask containing 63.0 ml of sterilised trypticase soy broth. The samples were mixed thoroughly to give a dilution of 1:10 and thereafter ten fold dilutions were made. The procedure adopted to examine samples is shown in Figure 1.

Figure 1. A simplified scheme for the identification of *B. cereus* (Kramer *et al.*, 1982).



Presumptive *Bacillus cereus* count :

The medium used was Mannitol Egg Yolk Polymyxin (MEYP) agar with phenol red (Mossel *et al*., 1967). Duplicate plates were used for inoculating 0.1 ml from preselected dilutions. The inoculum was spread as quickly as possible over the surface of the medium with the help of a sterile spreader. For each plate separate spreaders were used. The plates were left with lids on for about 15 minutes at ambient temperature for the inoculum to be absorbed into the agar.

The plates were incubated for 18 to 24 hours at 30°C. The period of incubation was extended further by 24 hours if no visible growth was observed.

Plates containing between 15 and 150 colonies, were selected for counting large pink (i.e., mannitol non-fermentor) and generally surrounded by a zone of precipitation (indicating the production of lecithinase). The average number of viable colonies per gram of sample was calculated as : Number of colonies x 10 x dilution factor. This gave presumptive *B. cereus* count.

Confirmatory *Bacillus cereus* count :

The number of colonies on MEYPA forming zone of precipitation multiplied by the reciprocal of the dilution yielded 'presumptive *B. cereus*' counts. Five colonies were selected and examined for morphological and biochemical characters. An isolate was confirmed as *B. cereus*, if it was Gram positive rod, motile, Lecitho-vitelline (LV), catalase

and Voges-Proskauer (VP) reactions positive, produced acid from glucose, but not from, mannitol, arabinose and xylose in Ammonium salt sugar (ASS). The number of confirmed B. cereus / g of the sample was estimated by the formula X/5 where X is the number of colonies confirmed as B. cereus out of 5 tested (Goepfert, 1976).

Enrichment method :

Twenty five gram of each sample of fried rice and chawmin was taken in a sterile flask. 50 to 100 ml of trypticase soy broth was added into the flask. The flasks were incubated at 30°C overnight. From these MEYPA was streaked with a 3 mm loop and incubated at 37°C overnight. Typical lecithinase positive, mannitol negative colonies were transferred to nutrient agar slants and incubated at 30°C overnight.

Isolation and identification :

On the basis of colony characters on MEYPA Gram's reaction, morphology and presence of spores, isolates were selected. The presumptive colonies were further tested for identification as per the method described by Cowan (1974).

Identification of bacterial isolates :

Following tests were done for species identification of Bacillus isolates from fried rice, chawmin and skimmed milk powder.

Gram's reaction: Cultures were stained by standard method of Gram's staining (Cruickshank et al., 1975) and results were noted for Gram's reaction, morphology and presence or absence of spores.

Spore staining: Smears were stained for spore by Schaeffer and Emulton's (1933) method as follows :

a) The slide was flooded with 5.0 per cent aqueous malachite green and steamed for 1 minutes.

b) The slide was washed under running water.

c) Counter stain was done with 0.5 per cent aqueous safranin for 15 seconds.

d) It was rinsed with water and air dried.

Bacterial bodies stained red and spores green.

Motility: Young (5-7 hours) broth cultures of the organism incubated at or below 30°C was examined by means of 'hanging drop' preparation using a high power dry objectives and reduced illumination.

Cytochrome oxidase test: The presence of cytochrome oxidase was determined by smearing culture from a solid medium on a filter paper impregnated with freshly made 1.0 per cent aqueous solution of N.N.N.N. tetramethyl-p-phenylene diamine dihydrochloride.

Oxidative and fermentative reaction: Isolates were tested for this reaction in Hugh and Leifson's medium. Two test tubes were

inoculated from each culture and one of them was sealed with sterile liquid paraffin. Breakdown of glucose (yellow colour) in both tubes indicated fermentation. Development of yellow colour only in open tube indicated oxidation.

Voges-Proskauer's test: Cultures were inoculated into glucose peptone broth and incubated for 48 hours. In this 1 ml 6.0 per cent alpha-naphthol solution and 0.4 ml of 40.0 per cent aqueous solution of potassium hydroxide were added. It was shaked well and observed for the appearance of red colour after 20 minutes.

Starch hydrolysis: Starch agar was inoculated with test culture and incubated for 5 days following which it was flooded with Lugol's iodine solution. The medium turned blue where starch was present and hydrolysis was indicated by clear colourless zones.

Nitrate reduction: Isolates were inoculated into nitrate broth and incubated at 37°C for 24 hours. Results were observed by adding 0.5 ml of 0.8 per cent sulphanilic acid solution in 5N-acetic acid and 4.0 drops of alpha-naphthol into culture. Appearance of red colour indicated nitrite i.e. NO_3 reduction. Finally zinc dust was added in tubes showing negative reactions to detect unreacted nitrate.

Gelatin hydrolysis: Gelatin (0.4 %) agar plates were inoculated with test cultures and incubated at 37°C for 3 days. After incubation the plates were flooded with 10 ml acid mercuric

chloride. Clear zones indicated areas of gelatin hydrolysis while white precipitate appeared where gelatin was present.

Citrate utilization: Isolates were inoculated into Simmon's citrate agar slant and incubated at 37°C upto 48 hours and reaction was noted.

Growth in 7.0 % Sodium chloride: A loopful of broth culture was inoculated in tubes containing nutrient broth with 7.0 per cent sodium chloride and incubated at 37°C for 7 days. Tubes were observed daily for growth.

Growth at 50°C and 65°C: Trypticase soy broth was inoculated with test culture and incubated at 50°C and 65°C for 48 hours. The tubes were examined for growth.

Casein hydrolysis: The cultures under test were streaked on skimmed milk agar plates and incubated at 37°C for 72 hours. Inoculated plates were flooded with 1.0 per cent hydrochloric acid or 10.0 per cent acetic solution for one minute. Colonies surrounded by clear zone indicated hydrolysis of casein and white precipitate around the colonies was regarded as unhydrolysed casein.

Lecithino-vitelline reaction (LV): Cultures were streaked on mannitol egg-yolk agar and incubated at 37°C for 24 hours. An opaque zone of precipitation around and beneath the colonies was indicative of lecithinase activity.

Acid from carbohydrates: Ammonium salt solution containing 1.0 per cent sugar such as glucose, arabinose, mannitol and salicin were inoculated and incubated at 37°C for 7 days. Yellow colour of the medium indicated acid production.

Serotyping with flagellar antigens:

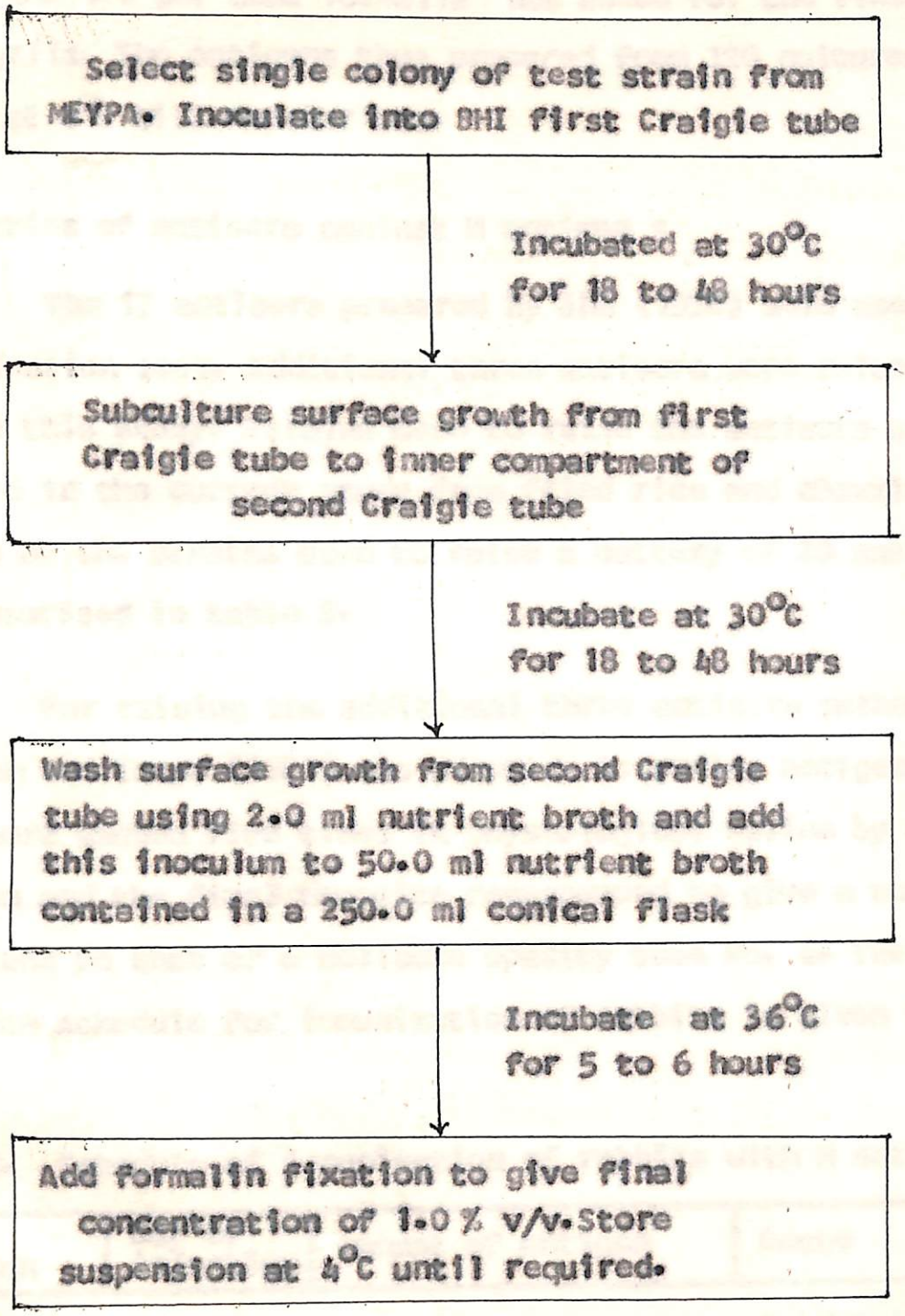
Preparation of flagellar antigens :

Flagellar antigen (H-antigen) was prepared following the method described by Gilbert and Taylor (1976) and Kramer et al. (1982) with slight modification (Figure 2). One hundred twenty cultures were used for raising the flagellar antigen.

The cultures were grown in nutrient broth. From this MEYPA was streaked and single colony was transferred to Brain Heart Infusion (BHI) broth. The first Craigie tube containing semi-solid agar was inoculated from growth in BHI. The tube was incubated at 30°C for 18 to 48 hours. Surface growth from first Craigie tube was transferred into second Craigie tube with the help of straight wire and incubated at 30°C for 18 to 48 hours. All the isolates (120) were given several passages through Craigie tube to get actively motile cultures.

Growth in the final Craigie tube was washed in 2 ml nutrient broth and transferred to 250 ml nutrient broth in a conical flask. This was incubated at 35°C for 8 hours. The flask was shaken manually at intervals of 30 minutes. One drop of this culture was taken on glass slide and checked for motility and absence of spores. The growth was centrifuged at 10,000 rpm in

Figure 2. Preparation of 'H' antigen suspension (Kramer *et al.*, 1982).



refrigerated centrifuge (Remi Model No. C24) for half an hour. The sediment was resuspended in 10 ml sterile physiological saline and 1.0 per cent formalin was added for the fixation of flagella. The antigens thus prepared from 120 cultures were stored at 4°C till further use.

Preparation of antisera against H antigen :

The 17 antisera prepared by Jha (1992) were used for agglutination test. Additional three antisera were raised and used in this study. Strains used to raise the antisera were isolated in the current study from fried rice and chawmin. Details of the strains used to raise a battery of 20 antisera are summarised in table 2.

For raising the additional three antisera method of Johnston and Torpe (1982) was adopted. According antigen suspensions were washed five times in physiological saline by centrifugation and the final deposits resuspended to give a turbidity equivalent to that of a Wellcome opacity tube No. 6. The injection schedule for immunization of rabbits is given in table 3.

Table 3. Schedule of immunization of rabbits with H antigen.

No. of injection	Day of injection	Amount of antigen	Route
1	0 day	1 ml + complete adjuvant 1 ml	Subcutaneous
2	15th day	1 ml + incomplete adjuvant 1 ml	Subcutaneous
3 (if required)	25 th day	1 ml	Subcutaneous

Test bleeding was done on 10th day of the second injection. If the titre was low then the third injection was given.

Table 2. Sources and other details of cultures used for the preparation of antisera.

Sl. No.	Isolate No.	Isolated from	Serotypes*
1.	1	Frankfurter	Non-typable
2.	2	Frankfurter	Non-typable
3.		Ready to eat sausage	Unknown
4.	6	Frankfurter	Non-typable
5.	7	Frankfurter	Non-typable
6.	8	Frankfurter	Non-typable
7.	11	Frankfurter	Non-typable
8.	13	Frankfurter	Non-typable
9.	14	Ready to cook frankfurter	Non-typable
10.	15	Flour	Non-typable
11.	17	Ready to cook frankfurter	Non-typable
12.	18	Flour	Non-typable
13.	21	Flour	Non-typable
14.	24	Flour	Non-typable
15.	FM	Flour	Unknown
16.	HSP_2	Heat treated spices	Unknown
17.	RE_2	Ready to eat sausage	Unknown
18.	5	Fried rice	Not done
19.	26	Fried rice	Not done
20.	75	Chawmin	Not done

* The isolate was serotyped at Food Hygiene Laboratory, Colindale, London.

Note: Antisera were raised against isolates Sl.No. 18 to 20 in this study. Rest were from Jha (1992).

Cultivation maintenance and characterization :

Cultivation and maintenance :

The isolates under test were inoculated into nutrient broth and incubated at 37°C overnight. The growth was streaked on MEYPA to check purity. Single colony was picked up and inoculated in semi-solid agar (SSA) in screw capped vials and stored at 4°C.

Biochemical characterization :

The cultures were rechecked for purity. Pure cultures were subjected to a set of biochemical tests (Table 4) for characterization compiled by Cowan (1974), Gibson and Gordon (1974) and Norris *et al.* (1981).

Table 4. Characters of *B. cereus* according to different workers.

Sl. No.	Characters	Sl. No.	Characters
1	Motility	6	Citrate utilization
2	LV reaction	7	Nitrate reduction
3	Catalase	8	Starch hydrolysis
4	Acid from Carbohydrate	9	Gelatin hydrolysis
	a) Glucose aerobic	10	Decomposition of casein
	b) Glucose anaerobic	11	Growth in anaerobic agar
	c) Arabinose		
	d) Mannitol	12	Growth in 7.0 % NaCl
	e) Xylose	13	Growth at 50°C and 65°C
5	Voges-Proskauer		

The study was further extended to test the ability to ferment 17 different sugars (Table 5). Ammonium salt sugar (ASS) medium described by Cowan (1974), was used to see the acid production from 17 different sugars. One per cent test sugar was inoculated. These were incubated at 37°C and observed for seven days.

Table 5. Sugars used in the fermentation study.

Sl. No.	Sugars	Sl. No.	Sugars
1.	D-Fructose	10.	Arabinose
2.	Sucrose	11.	Salicin
3.	L-Rhamnose	12.	D-Sorbitol
4.	Mannose	13.	Meso-inositol
5.	Cellobiose	14.	Glycogen
6.	D-Galactose	15.	Dulcitol
7.	Lactose	16.	Melibiose
8.	Mannitol	17.	D-Glucose
9.	D-Xylose		

Agglutination test :

The test was carried out in microtitre plate, following the method of Jha (1992) which is a modification of these described by Cruickshank et al. (1975), Gilbert and Taylor (1976).

In all the wells 50 µl of physiological saline was taken with the help of micropipette (Dynatech). In separate tube 1:10 dilution was made by mixing 50 µl of antisera into 0.45 ml of physiological saline. From this tube 50 µl of diluted serum (1:10) was transferred into each well of column one of rows A to H of microtitre agglutination plate to get a dilution of 1:20. After thorough mixing, 50 µl of diluted serum was transferred from column 1 into column 2, and mixed to get a two fold dilution and similarly continued upto column 11. The column 12 was kept as control for respective antigens. Each well in the rows from A to H was charged with eight different antigens. After this the plate was incubated at 37°C overnight.

Agglutination was read in mirror against reflected light. In this way each serum was tested with 120 antigens.

RESULTS

Enumeration of *Bacillus cereus* in chawmin, fried rice and skimmed milk powder :

Viable counts of *B. cereus* and bacteria other than *B. cereus* were made in a total number of 226 samples consisting of chawmin (100), fried rice (100) and skimmed milk powder (26). Viable count has been expressed in $\log_{10}$ /gm.

Twenty three samples of chawmin were found positive for *B. cereus* (Table 6). The remaining 77.0 per cent of samples were negative. The number of B. cereus ranged from zero to 3.9193 with an average of 0.6453 ± 0.1225/g. Seventy four samples (74.0 %) were found contaminated with bacteria other than *B. cereus*. The counts of such bacteria ranged from zero to 5.4771 with an average of 2.8606 ± 0.1852/g (Figure 3).

The results of counts of *B. cereus* and bacteria other than *B. cereus* in fried rice are presented in Table 7.

Altogether 24 (24.0 %) samples of fried rice were found positive for *B. cereus*. *B. cereus* counts in these samples ranged from zero to 5.4564 with an average of 0.7395 ± 0.1401/g. A total of 76 (76.0 %) samples were found free from *B. cereus* contamination. For bacteria other than *B. cereus*, 66 (66.0 %) samples were positive. Counts of these ranged from zero to 5.6989 with an average of 2.5543 ± 0.1990/g. Thirty four samples were free from the bacteria other than *B. cereus* (Figure 3). The number of samples indentified as positive for *B. cereus* and later confirmed to be so was the same i.e. 24. Also the same samples were identified by both presumptive and confirmatory tests as positive for *B. cereus*.

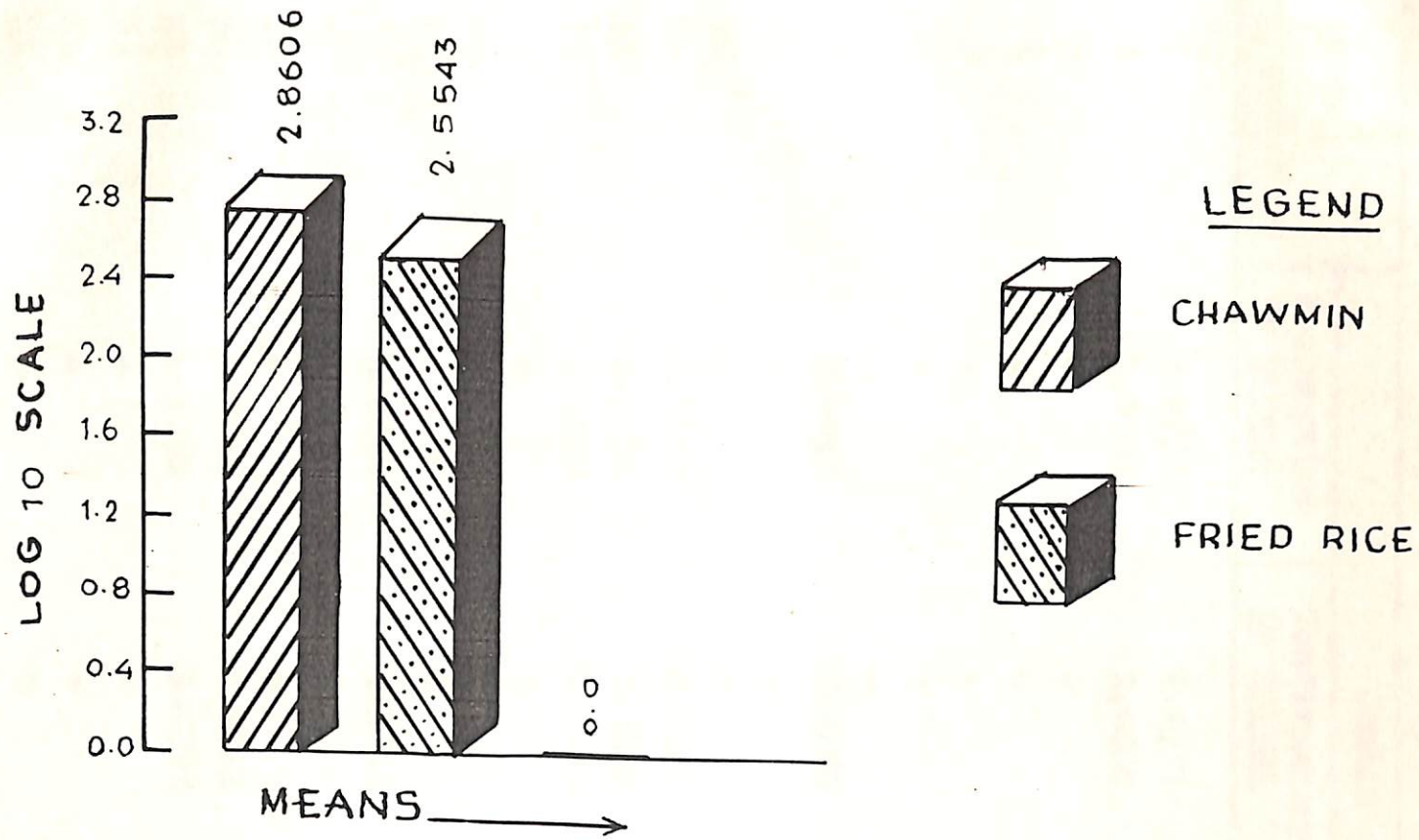


FIG.3 BAR DIAGRAMES SHOWING AVERAGE COUNTS OF BACTERIA OTHER THAN B. Cereus

Table 6. Presumptive and confirmatory counts of *B. cereus* and bacteria other than *B. cereus* in chawmin /gm in $\log_{10}$ scale.

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
1	2.4771	2.4771	-
2	2.0000	2.0000	2.7781
3	-	-	5.2430
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	2.9031	2.8062	-
9	-	-	-
10	-	-	-
11	2.0000	2.0000	3.1461
12	2.7781	2.6812	5.3522
13	2.0000	2.0000	3.4150
14	-	-	-
15	-	-	-
16	-	-	-
17	3.1461	2.9243	-
18	-	-	4.0792
19	-	-	3.9542
20	3.0792	2.9823	2.6990
21	3.3010	3.0792	4.0000
22	-	-	5.3979
23	-	-	3.1461
24	-	-	3.7781
25	-	-	3.6020
26	-	-	3.2553
27	-	-	5.1761
28	2.0000	2.0000	2.3010

Contd. ..../-

Table 6. Contd. .../-

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
29	-	-	3.9031
30	-	-	4.0000
31	-	-	3.7482
32	-	-	5.3225
33	-	-	4.1139
34	-	-	3.6232
35	-	-	3.6812
36	-	-	4.5315
37	-	-	4.1139
38	-	-	3.8451
39	-	-	3.7781
40	-	-	5.2553
41	-	-	5.2430
42	-	-	3.0000
43	2.0000	2.0000	2.4771
44	-	-	5.0969
45	3.0000	2.9031	4.7924
46	-	-	5.3979
47	-	-	4.1461
48	3.0000	3.0000	-
49	-	-	3.5051
50	-	-	3.3222
51	-	-	5.1761
52	-	-	5.0000
53	-	-	5.4771
54	-	-	3.9445
55	-	-	3.0000
56	-	-	5.4393
57	-	-	3.6990
58	-	-	5.3010

Contd. .../-

Table 6. Contd. ...

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
59	-	-	3.2041
60	-	-	3.2304
61	-	-	3.7324
62	-	-	4.0569
63	-	-	3.7464
64	-	-	3.4471
65	-	-	4.3802
66	-	-	4.0000
67	-	-	3.0792
68	-	-	2.9542
69	-	-	4.0792
70	2.0000	2.0000	3.3222
71	-	-	3.3010
72	-	-	3.5315
73	-	-	3.1139
74	2.0000	2.0000	-
75	3.0000	3.0000	-
76	-	-	-
77	4.0128	3.9193	-
78	3.5911	3.3692	-
79	4.0000	3.9031	-
80	3.6020	3.6020	-
81	3.3010	3.2041	-
82	3.7781	3.6812	-
83	-	-	-
84	-	-	2.7781
85	-	-	-
86	-	-	-
87	-	-	3.2041
88	-	-	3.2304

Contd. ...

Table 6. Contd. .../-

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
89	-	-	3.6020
90	3.0000	3.0000	-
91	-	-	2.8451
92	-	-	4.5185
93	-	-	3.6990
94	-	-	3.3979
95	-	-	3.3979
96	-	-	3.1761
97	-	-	4.3979
98	-	-	4.0000
99	-	-	2.7781
100	-	-	2.6020
Average ± SE	0.6597 ±0.1256	0.6453 ±0.1225	2.8606 ±0.1852
Minimum	0.0000	0.0000	0.0000
Maximum	4.0128	3.9193	5.4771
No. of positive samples	23	23	74
Positivity (%)	23.00	23.00	74.00

Table 7. Presumptive and confirmatory counts of *B. cereus* and bacteria other than *B. cereus* in fried rice/gm in $\log_{10}$ scale.

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
1	2.6020	2.5051	-
2	2.3010	2.3010	-
3	-	-	5.6989
4	-	-	-
5	2.0000	2.0000	-
6	-	-	-
7	-	-	-
8	2.7781	2.6812	-
9	-	-	-
10	-	-	-
11	2.7781	2.6812	5.4393
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	2.3010	2.3010	3.2304
19	3.6020	3.5051	5.2787
20	-	-	-
21	-	-	-
22	2.0000	2.0000	3.3010
23	4.8692	4.6474	-
24	-	-	5.0000
25	-	-	3.3424
26	-	-	2.7781
27	-	-	5.1461
28	-	-	-

Contd. ...

Table 7. Contd. .../-

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
29	2.0000	2.0000	3.3222
30	-	-	4.0792
31	-	-	5.2430
32	2.3010	2.3010	3.6532
33	3.0000	2.9031	4.0792
34	-	-	3.7482
35	-	-	3.0792
36	-	-	3.3424
37	3.6990	3.6020	5.0792
38	-	-	3.2304
39	-	-	3.2304
40	-	-	5.3522
41	-	-	5.3979
42	-	-	-
43	3.0000	3.0000	3.4771
44	-	-	5.4771
45	-	-	5.3010
46	-	-	3.7482
47	-	-	3.5682
48	-	-	3.1461
49	-	-	2.3010
50	-	-	5.0792
51	-	-	5.1139
52	-	-	3.2553
53	-	-	3.3222
54	-	-	3.6020
55	-	-	3.3541
56	-	-	5.1761
57	-	-	5.2430
58	-	-	3.3010

Contd. .../-

Table 7. Contd. ...../-

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
59	-	-	3.0000
60	-	-	-
61	-	-	3.2553
62	-	-	3.3010
63	-	-	4.7160
64	-	-	3.3424
65	-	-	3.2304
66	-	-	3.1139
67	-	-	4.1139
68	-	-	3.3424
69	-	-	3.5185
70	3.4771	3.4771	4.0000
71	-	-	3.8865
72	-	-	-
73	-	-	-
74	-	-	-
75	5.6128	5.4564	-
76	4.6627	4.5658	-
77	3.7781	3.6812	-
78	4.2787	3.7160	-
79	3.6990	3.6990	-
80	4.1461	3.9243	-
81	-	-	-
82	-	-	2.6990
83	-	-	-
84	3.0000	3.0000	-
85	-	-	3.8451
86	-	-	2.7781
87	-	-	4.0792
88	2.0000	2.0000	5.1761

Contd. ...../-

Table 7. Contd. .../-

Sl.No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
89	-	-	2.7781
90	-	-	2.6020
91	3.0000	3.0000	5.1761
92	-	-	3.0000
93	-	-	3.5441
94	-	-	3.3010
95	-	-	4.6990
96	-	-	3.5441
97	-	-	3.1761
98	-	-	-
99	-	-	3.0000
100	-	-	2.6990
Average ± SE	0.7688 ±0.1457	0.7394 ±0.1400	2.5543 ±0.1990
Minimum	0.0000	0.0000	0.0000
Maximum	5.6128	5.4564	5.6989
No. of positive samples	24	24	66
Positivity (%)	24.00	24.00	66.00

The results of B. cereus and bacteria other than B. cereus counts in skimmed milk powder has been given in Table 8.

Eighteen samples (69.23 %) were found to be contaminated with B. cereus and the counts ranged from zero to 3.6812 with an average of 1.9386 ± 0.2736/g. Altogether 30.76 per cent samples were found free from B. cereus contamination. The same samples and also the same number of samples were found contaminated with B. cereus by both the presumptive and confirmatory tests.

It is interesting to note that none of the samples of skimmed milk powder was found positive for bacteria other than B. cereus (Figure 3).

In order to test the reliability of presumptive identification of B. cereus percentage of colonies taken from MEYPA and later confirmed as B. cereus in each sample of the three foods examined were tabulated in Table 9. With the help of characters of B. cereus colonies described, it was possible to identify this in 60.0 to 100.0 per cent cases with an overall average of 88.0 ± 1.7431 per cent.

The average presumptive and confirmatory counts of B. cereus in the three food samples were shown in Figure 4 .

The distribution of chawmin, fried rice and skimmed milk powder samples according to level of contamination determined by presumptive counts of B. cereus has been presented in Table 10. None of the samples of the three foods examined showed presumptive B. cereus counts less than $\angle$2.0/g. In

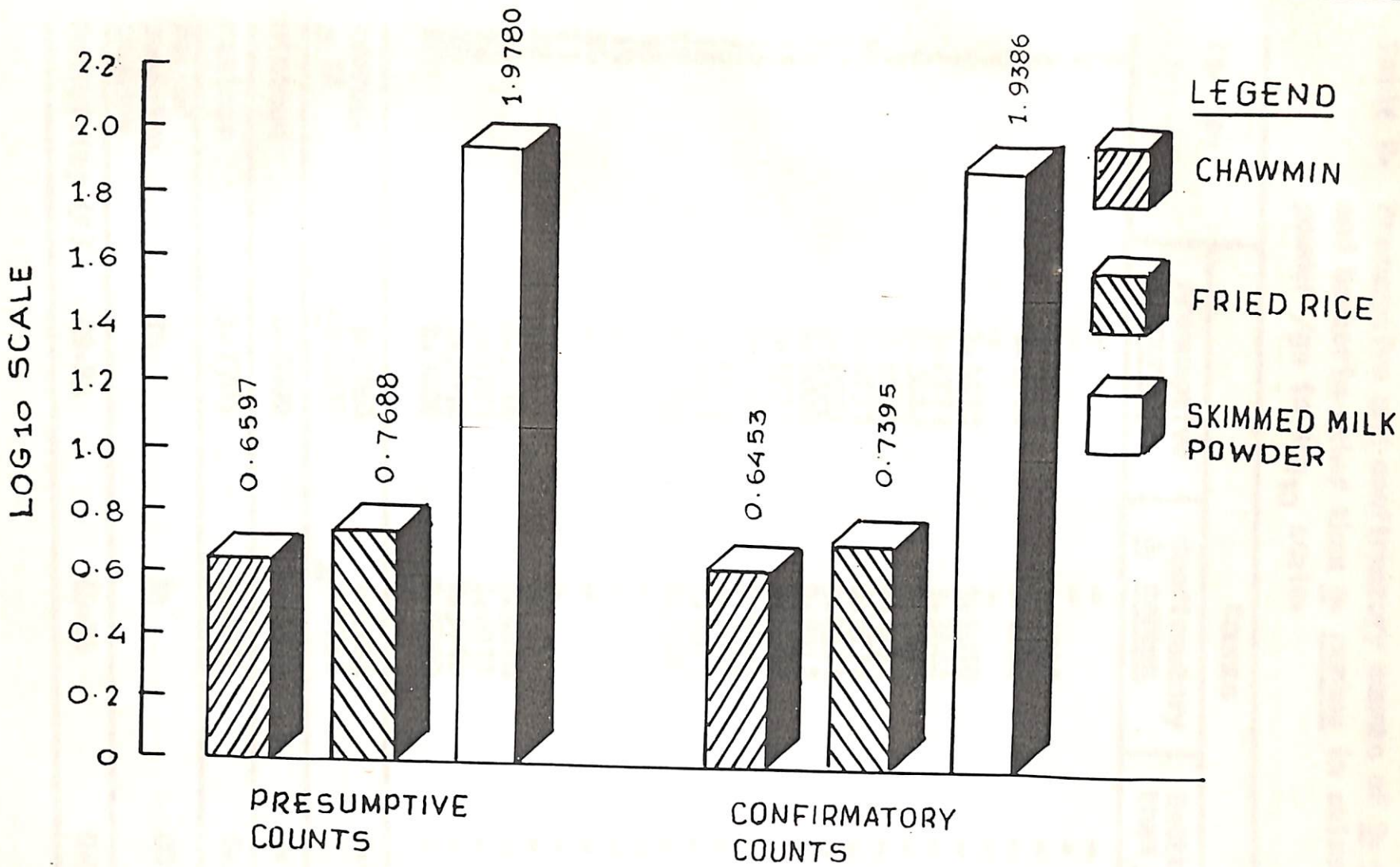


FIG. 4 BAR DIAGRAMES SHOWING MEAN COUNTS OF BACILLUS CEREUS

Table 8. Presumptive and confirmatory counts of *B. cereus* and bacteria other than *B. cereus* in skimmed milk powder /gm in $\log_{10}$ scale.

Sl. No.	Counts		
	Presumptive *B. cereus*	Confirmatory *B. cereus*	Bacteria other than *B. cereus*
1	-	-	-
2	-	-	-
3	2.4771	2.4771	-
4	2.6020	2.6020	-
5	-	-	-
6	2.3010	2.3010	-
7	3.4771	3.3802	-
8	3.6020	3.5051	-
9	2.6990	2.4771	-
10	2.4771	2.4771	-
11	3.2553	3.0334	-
12	3.0000	2.9031	-
13	3.4771	3.3802	-
14	3.0000	3.0000	-
15	3.7781	3.6812	-
16	2.9031	2.9031	-
17	2.0000	2.0000	-
18	-	-	-
19	-	-	-
20	-	-	-
21	-	-	-
22	-	-	-
23	2.3010	2.3010	-
24	3.7781	3.6812	-
25	2.3010	2.3010	-
26	2.0000	2.0000	-
Average ± SE	1.9780 ±0.2807	1.9386 ±0.2736	0.0000
Minimum	0.0000	0.0000	0.0000
Maximum	3.7781	3.6812	0.0000
No. of positive samples	18	18	00
Positivity (%)	69.23	69.23	0.00

Table 9. Percentage of colonies from presumptive counts confirmed as *B. cereus*.

Sl.No.	Per cent colonies confirmed as *B. cereus* in samples of		
	Chawmin	Fried rice	Skimmed milk powder
1	100	80	100
2	100	100	100
3	80	100	100
4	100	80	80
5	80	80	80
6	100	100	60
7	60	80	100
8	80	100	60
9	60	60	80
10	100	100	80
11	100	100	100
12	80	80	80
13	100	80	100
14	100	100	100
15	100	100	100
16	100	60	80
17	80	80	100
18	60	80	100
19	80	80	-
20	100	100	-
21	80	60	-
22	80	100	-
23	100	100	-
24	-	100	-
Mean ± SE	87.830 ± 3.012	87.500 ± 2.902	88.890 ± 3.322
Minimum (%)	60	60	60
Maximum (%)	100	100	100
Overall (%)	88.00 ± 1.743		

other words, those positive had counts $\neg$ 2.0/g. While ten (10.0 %) samples of chawmin had counts between 2.00 to 3.00 and 11 (11.0 %) between 3.00 and 4.00/g, only 2(2.0 %) showed counts ranging from 4.00 to 5.00/g. A total of 77 (77.0 %) samples were found negative for B. cereus counts.

Table 10. Distribution of samples of chawmin, fried rice and skimmed milk powder according to level of contamination with B. cereus (presumptive count).

Range	Chawmin	Fried rice	Skimmed milk powder
$\angle$ 2.0	77(77.00)	76(76.00)	8(30.77)
2.0-3.0	10(10.00)	10(10.00)	10(38.46)
3.0-4.0	11(11.00)	9(9.00)	8(30.77)
4.0-5.0	2(2.00)	4(4.00)	0
5.0-6.0	0	1(1.00)	0
Total	100	100	26

Figure in parenthesis shows the percentages.

In case of fried rice 24(24.0 %) samples were found contaminated with B. cereus. The break-up of these according to level of contamination is as follows : Ten (10.0 %) ranging from 2.00 to 3.00/g, 9(9.0 %) 3.00 to 4.00/g, 4(4.0 %) 4.00 to 5.00/g and only one (1.0 %) between 5.00 to 6.00/g.

In case of skimmed milk powder, 10(38.46 %) had counts of B. cereus ranging from 2.00 to 3.00/g and 8(30.77 %) 3.00 to 4.00/g.

Of the three foods examined, relatively higher percentage of skimmed milk powder samples were found contaminated with B. cereus.

The distribution of chawmin, fried rice and skimmed milk powder samples according to level of confirmatory B. cereus counts has been tabulated in Table 11.

Table 11. Distribution of samples of chawmin, fried rice and skimmed milk powder according to level of contamination with B. cereus (confirmatory count).

Range	Chawmin	Fried rice	Skimmed milk powder
∠2.0	77(77.00)	76(76.00)	8(30.76)
2.0-3.0	13(13.00)	11(11.00)	11(42.30)
3.0-4.0	10(10.00)	10(10.00)	7(26.92)
4.0-5.0	0	2(2.00)	0
5.0-6.0	0	1(1.00)	0
Total	100	100	26

Figure in parenthesis shows the percentages.

Thirteen (13.0 %) samples of chawmin showed counts ranging from 2.00 to 3.00/g while 10(10.0 %) fell in the range of 3.00 to 4.00/g. The level of B. cereus contaminating fried rice ranged from 2.00 to 6.00/g. The distribution of samples were as follows.

Eleven (11.0 %) showed counts in the range of 2.00 to

3.00/g, 10(10.0 %) 3.00 to 4.00/g, 2(2.0 %) 4.00 to 5.00/g and one (1.0 %) between 5.00 to 6.00/g.

In majority of the samples of skimmed milk powder (42.30 %) B. cereus counts ranged from 2.00 to 3.00/g, while in 26.92 per cent it was between 3.00 to 4.00/g.

Figure 5 presents a comparative view of the levels of contamination with B. cereus determined as presumptive and confirmatory counts in three foods examined. In figure 6 level of contamination with bacteria other than B. cereus is shown.

In Table 12 are summarised the distribution of samples of chawmin and fried rice accordingly to the levels of contamination with bacteria other than B. cereus. All the samples of skimmed milk powder were negative for bacteria other than B. cereus. Twenty six and 34.0 per cent of chawmin and fried rice samples, respectively were considered negative for these bacteria (counts being $\angle$ 2.0/g). Majority i.e. 36.0 per cent of chawmin and 38.0 per cent fried rice had counts of these bacteria between 3.00 to 4.00/g. These two foods did not differ significantly with respect of contamination with bacteria other than B. cereus at any of the five different levels of counts studied as evidenced by the normal deviate test shown in Table 13.

It is interesting to note that 100.0 per cent samples of skimmed milk powder examined were found negative for bacteria other than B. cereus.

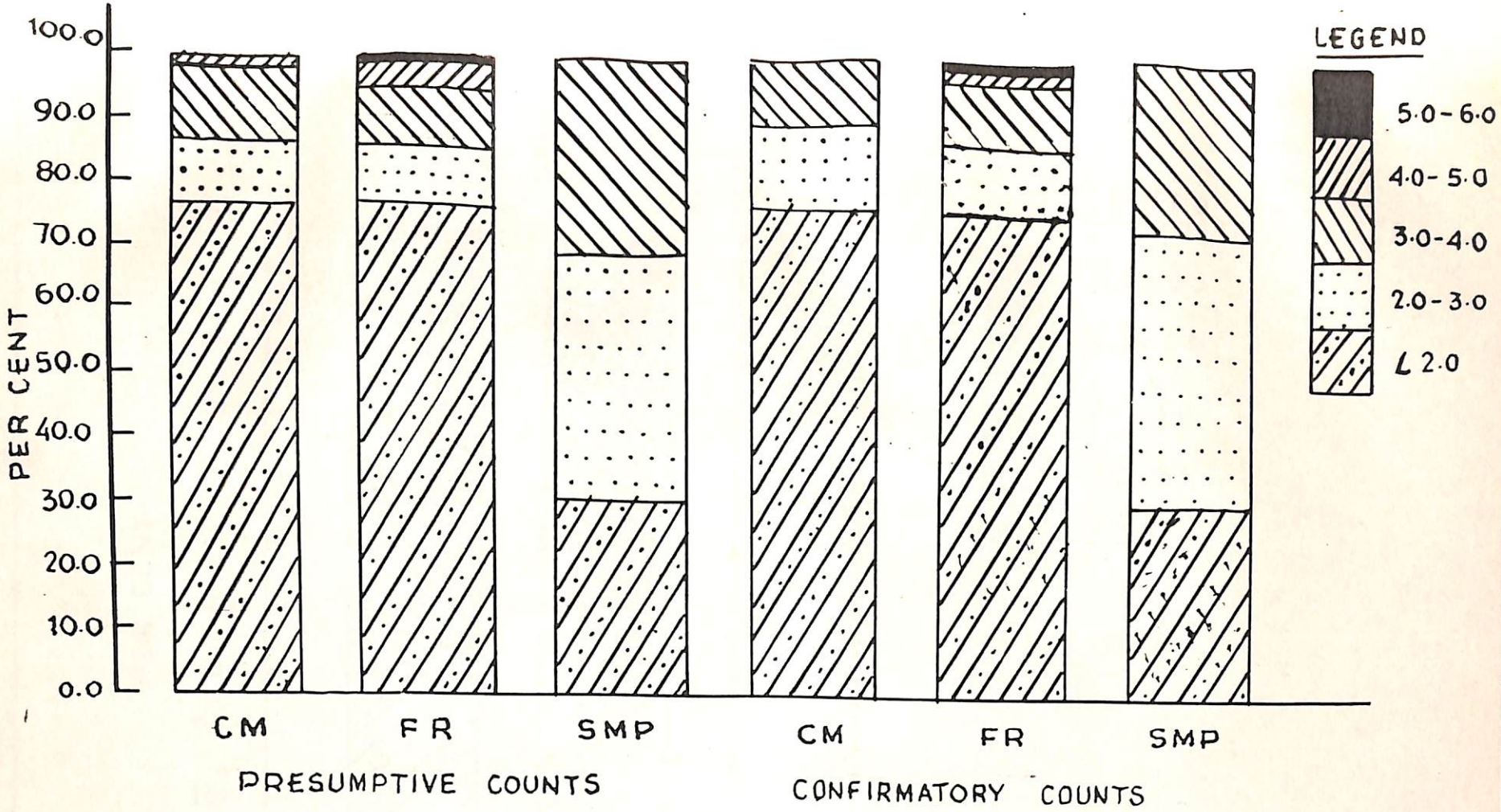


FIG. 5 DISTRIBUTION OF SAMPLES OF CHAWMIN (CM) FRIED RICE (FR) AND SKIMMED MILK POWDER (SMP) ACCORDING TO LEVEL OF CONTAMINATION WITH *B. Cereus*

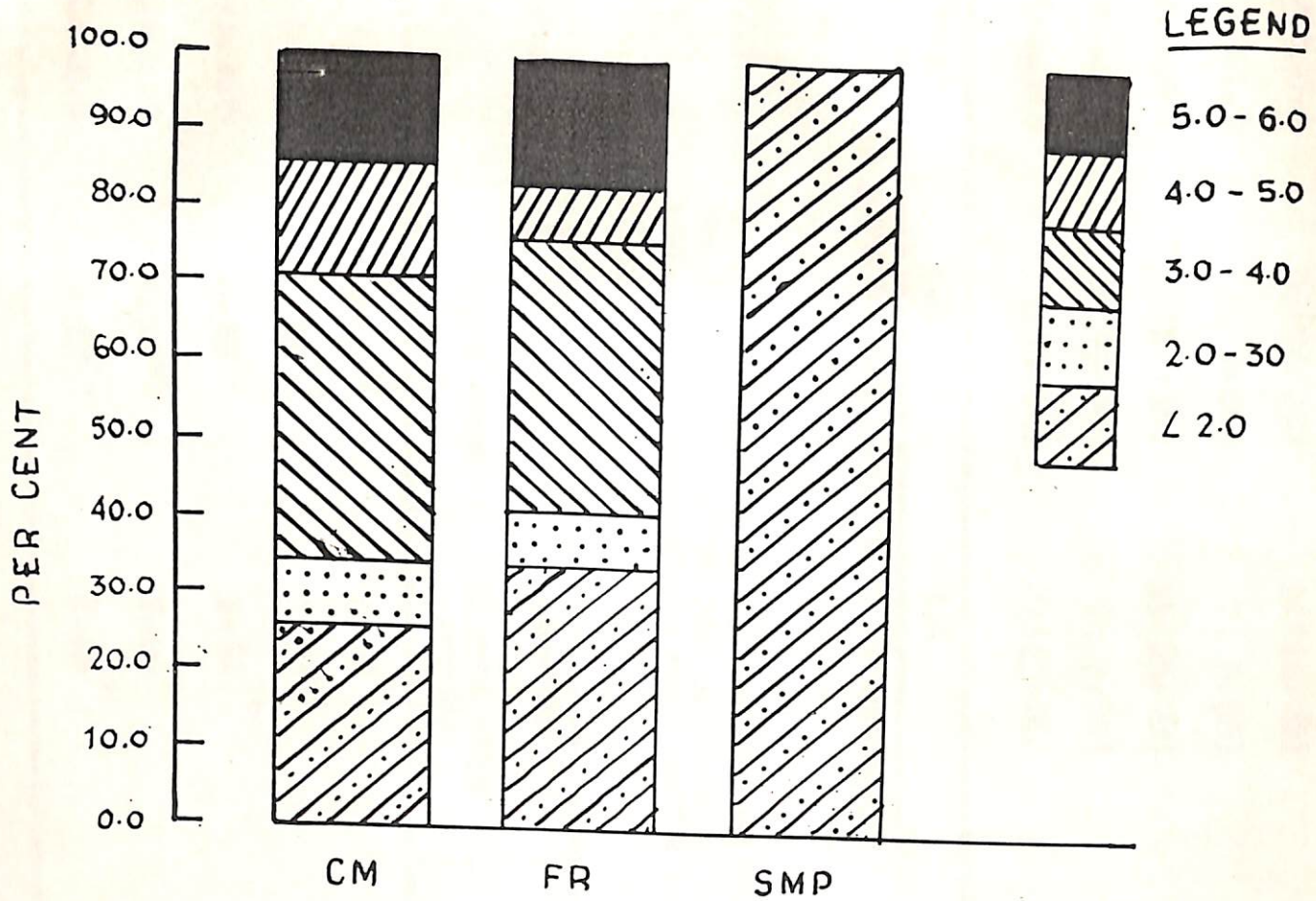


FIG. 6 DISTRIBUTION OF SAMPLES OF CHAWMIN (CM) FRIED RICE (FR) AND SKIMMED MILK POWDER (SMP) ACCORDING TO LEVEL OF CONTAMINATION WITH BACTERIA OTHER THAN *B. Cereus*

Table 12. Distribution of samples of chawmin, fried rice and skimmed milk powder according to level of contamination with bacteria other than *B. cereus*.

Range	Chawmin	Fried rice	Skimmed milk powder
<2.0	26(26.00)	34(34.00)	26(100.00)
2.0-3.0	9(9.00)	7(7.00)	0
3.0-4.0	36(36.00)	35(35.00)	0
4.0-5.0	15(15.00)	7(7.00)	0
5.0-6.0	14(14.00)	17(17.00)	0
Total	100	100	26

Figure in parenthesis shows the percentages.

Table 13. Normal deviate test to compare difference among chawmin, fried rice and skimmed milk powder for level of contamination with bacteria other than *B. cereus*.

Range	Chawmin Vs Fried rice	Chawmin Vs Skimmed milk powder	Fried rice Vs Skimmed milk powder
<2.0	1.23^{NS}	6.84^{**}	0.73^{NS}
2.0-3.0	0.52^{NS}	1.61^{NS}	1.46^{NS}
3.0-4.0	0.15^{NS}	3.64^{**}	3.54^{**}
4.0-5.0	1.81^{NS}	2.09^{*}	1.46^{NS}
5.0-6.0	0.60^{NS}	2.03^{*}	2.30^{*}

* $P < 0.05$; ** $P < 0.01$; NS = Non-significant.

Based on the presumptive counts of B. cereus 77.0, 76.0 and 30.77 per cent samples of chawmin, fried rice and skimmed milk powder, respectively, were negative. All negative samples were placed in the range of $\angle$2.0/g contamination. The level of contamination of $\angle$4.0/g of samples was observed in 98.0, 95.0 and 100.0 per cent samples of chawmin, fried rice and skimmed milk powder, respectively.

Comparisons were made among chawmin, fried rice and skimmed milk powder in all possible combinations for contamination with B. cereus (presumptive counts). Normal deviate tests (Table 14) revealed that the incidence of contamination with B. cereus was significantly higher in skimmed milk powder than chawmin and fried rice in the level of 2.00 to 3.00 and 3.00 to 4.00/g of sample. Normal deviate tests also suggested that the differences in incidence of higher level of contamination ($\neg$ 4.00/g) in skimmed milk powder, chawmin and fried rice were not significant. The difference in incidence of contamination with B. cereus between chawmin and fried rice was not significant in any range of contamination under study.

It was observed that 100.0, 97.0 and 100.0 per cent of samples of chawmin, fried rice and skimmed milk powder had counts of confirmed B. cereus upto $\angle$4.00/g (Table 11). The normal deviate value to compare the difference among the three foods for contamination with confirmed B. cereus in different levels of counts are presented in Table 15. Chawmin and fried rice samples did not differ significantly with respect to

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Table 14. Normal deviate test to compare difference among chawmin, fried rice and skimmed milk powder for contamination with B. cereus (presumptive count).

Range	Chawmin Vs Fried rice	Chawmin Vs Skimmed milk powder	Fried rice Vs Skimmed milk powder
<2.0	0.17^{NS}	4.47^{**}	4.37^{**}
2.0-3.0	0.00^{NS}	3.53^{**}	3.54^{**}
3.0-4.0	0.47^{NS}	2.52^{*}	2.94^{**}
4.0-5.0	0.83^{NS}	0.73^{NS}	1.07^{NS}
5.0-6.0	1.00^{NS}	0.00^{NS}	0.51^{NS}

* $P < 0.05$; ** $P < 0.01$; NS = Non-significant.

Table 15. Normal deviate test to compare difference among chawmin, fried rice and skimmed milk powder for contamination with B. cereus (confirmatory count).

Range	Chawmin Vs Fried rice	Chawmin Vs Skimmed milk powder	Fried rice Vs Skimmed milk powder
<2.0	0.17^{NS}	4.47^{**}	4.37^{**}
2.0-3.0	0.50^{NS}	3.329^{**}	3.789^{**}
3.0-4.0	0.00^{NS}	2.29^{*}	2.29^{*}
4.0-5.0	2.02^{*}	0.00^{NS}	0.72^{NS}
5.0-6.0	2.04^{*}	0.00^{NS}	0.51^{NS}

* $P < 0.05$; ** $P < 0.01$; NS = Non-significant.

contamination with B. cereus in the range of /2.0, 2.0-3.0 and 3.0-4.0/g. Samples of chawmin and skimmed milk powder and also fried rice and skimmed milk powder differ significantly, on the other hand, in the same ranges of contamination with B. cereus.

Analysis of variance (Table 16) revealed significant difference for average counts of B. cereus (presumptive and confirmatory) and bacteria other than B. cereus in chawmin, fried rice and skimmed milk powder. Critical difference test indicated that the average counts of B. cereus (presumptive) per gram was significantly higher in skimmed milk powder (1.9780 ± 0.2807) than chawmin (0.6597 ± 0.1256) and fried rice (0.7688 ± 0.1459) which did not differ significantly between themselves. Similar trend was observed for confirmatory counts of B. cereus.

Critical difference test (Table 17) further revealed non-significant difference for average count of bacteria other than B. cereus, chawmin and fried rice. All skimmed milk powder samples were negative for bacteria other than B. cereus. Statistically it was confirmed that bacteria other than B. cereus counts/g was significantly higher in chawmin and fried rice than skimmed milk powder.

No significant difference in level of contamination, percentage positivity and range of bacterial contamination with B. cereus was observed in samples of chawmin and fried rice.

Table 16. Analysis of variance showing effects of chawmin, fried rice and skimmed milk powder on *B. cereus* and bacteria other than *B. cereus*.

Source of variation	d.f.	*Bacillus cereus* (presumptive count)		*Bacillus cereus* (confirmatory count)		Bacteria other than *B. cereus*	
		M.S.	F	M.S.	F	M.S.	F
Between foods	2	18.6715	9.97**	18.0883	10.30**	86.6753	26.41**
Error	223	1.8723		1.7560		3.2818	

** $P < 0.01$

Table 17. Mean with S.E. for counts $\log_{10}$ scale of *B. cereus* and bacteria other than *B. cereus*.

Organisms	Chawmin	Fried rice	Skimmed milk powder
B. cereus (presumptive count)	0.6570^{b} ±0.1256	0.7688^{b} ±0.1459	1.9780^{a} ±0.2807)
B. cereus (confirmatory count)	0.6453^{b} ±0.1225	0.7395^{b} ±0.1401	1.9386^{a} ±0.2736
Bacteria other than *B. cereus*	2.8606^{a} ±0.1852	2.5543^{a} ±0.1990	0.0000^{b}

Mean values bearing same superscripts in a row did not differ significantly.

It may be because both are of cereal origin and were obtained from the same fast food restaurant, the hygienic standards of which were common to both the foods.

Per cent positivity for B. cereus was significantly higher (69.23 %) in skimmed milk powder than in chawmin and fried rice (52.0 %) and 60.0 % respectively) which did not differ significantly between themselves. The difference for per cent negative samples in 3 groups was not significant (Table 18).

Normal deviate test also indicated that the per cent positivity by direct plating for B. cereus was significantly higher in skimmed milk powder (69.23 %) than chawmin and fried rice (23.0 % and 24.0 %). The difference between chawmin and fried rice was not significant. Opposite trend was observed for per cent negativity by direct plating.

Per cent positivity for B. cereus by enrichment was highest in fried rice (47.37 %) followed by chawmin (37.66 %) and skimmed milk powder (0 %). The difference between chawmin and fried rice was not significant. Opposite trend was noticed for per cent negativity by enrichment. Significant highest per cent negativity (100.0 %) was observed in skimmed milk powder.

Per cent positivity for bacteria other than B. cereus was highest in chawmin (74.0 %) followed by fried rice (66.0 %) and skimmed milk powder (0 %).

Table 18. Normal deviate test to compare chawmin, fried rice and skimmed milk powder on the basis of per cent positive for B. cereus and bacteria other than B. cereus.

Source	No. of samples examined	Enrichment for B. cereus		Direct plating for B. cereus		Total samples for B. cereus		Bacteria other than B. cereus	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Chawmin	100	29/77 (37.66)[a]	48/77 (62.34)[a]	23 (23.00)	77 (77.00)	52 (52.00)[a]	48 (48.00)[a]	74 (74.00)	26 (26.00)[b]
Fried rice	100	36/76 (47.39)[a]	40/76 (52.63)[a]	24 (24.00)[b]	76 (76.00)	60 (60.00)[a]	40 (40.00)[a]	66 (66.00)	34 (34.00)[b]
Skimmed milk	26	0/8 (0)[b]	8/8 (100.00)[a]	18 (69.23)[a]	8 (30.77)[b]	18 (69.23)[a]	8 (30.77)	0 (0)[b]	26 (100.00)[a]

Figures in parenthesis indicates the percentages.

The difference between chawmin and fried rice was not significant ($P > 0.05$). Significantly higher per cent negativity for bacteria other than *B. cereus* was observed in skimmed milk powder (100.0 %) than chawmin (26.0 %) and fried rice (34.0 %) which did not differ significantly between themselves.

Difference between percentage positivity for *B. cereus* in samples of chawmin and fried rice was non-significant while significantly more samples of skimmed milk powder were found positive. A comparison of the two methods detection of *B. cereus* viz. direct plating Vs enrichment was made. Normal deviate tests revealed significant difference between positivity for *B. cereus* by direct plating and enrichment method in chawmin ($P < 0.05$), fried rice ($P < 0.01$) and skimmed milk powder. Per cent positivity by enrichment was significantly higher than direct plating in chawmin and fried rice. However, opposite trend was observed in skimmed milk powder indicating higher per cent positivity by direct plating than by enrichment test.

Isolation of *Bacillus cereus* :

All the 226 samples comprising of chawmin (100), fried rice (100) and skimmed milk powder (26) were processed by direct plating and enrichment methods for the isolation of *B. cereus*. A total of 120 isolates from the three foods examined were considered as *B. cereus*. The results of different tests carried out for identification are summarised up in Table 19. These isolates were distributed as 45, 56 and 19 from chawmin, fried rice and skimmed milk powder, respectively.

Table 19. Characters of *Bacillus cereus* isolates.

Sl. No.	Characters	Reaction observed	Compared with those reported by
1.	Motility	+	3
2.	Lecithino-vitelline reaction	+	2,3
3.	Catalase	+	1,3
4.	Acid from carbohydrates		
	Glucose aerobic	+	1,3
	Glucose anaerobic	+	3
	Arabinose	-	2,3
	Mannitol	-	2,3
5.	Voges-Praskauer reaction	+	1,3
6.	Citrate utilization	+	3
7.	Nitrate reduction	+	1,2,3
8.	Hydrolysis of starch	+	1,3
9.	Hydrolysis of gelatin	+	3
10.	Decomposition of casein	+	1,3
11.	Growth in		
	Anaerobic agar	+	1
	7.0 % NaCl	+	1
	at 65°C	-	3

1. Norris *et al.* (1981)
2. Gibson and Gordon (1974)
3. Cowan (1974)

The samples which gave negative results by direct plating were examined by enrichment method.

Morphological, cultural and biochemical characters of B. cereus isolates :

The 120 isolates from fried rice (56), chaumin (45) and skimmed milk powder (19) were studied for specific identification.

All these isolates exhibited certain common morphological, cultural and biochemical characters as shown in Table 19. The study was further extended to test fermentation of as many as 17 different sugars. All isolates were similar with respect to fermentation of 10 sugars. However, these were found variable with respect to fermentation of rhamnose, D-sorbitol, arabinose, xylose, salicin and glycogen. The details of the fermentation reaction of isolates of B. cereus isolated from chaumin, fried rice and skimmed milk powder are presented in Tables 20, 21 and 22 respectively.

Out of 120 isolates, 21.66 per cent fermented xylose, 67.50 per cent salicin and 85.00 per cent cellobiose. Sixteen isolates fermented both salicin and xylose. Ten isolates were xylose positive and salicin negative (Figure 7).

According to the standard of PHLS, Colindale to declare an isolate as fermenting (80 - 100 % positive) or non-fermenting (0 - 20 % negative) with respect to a character

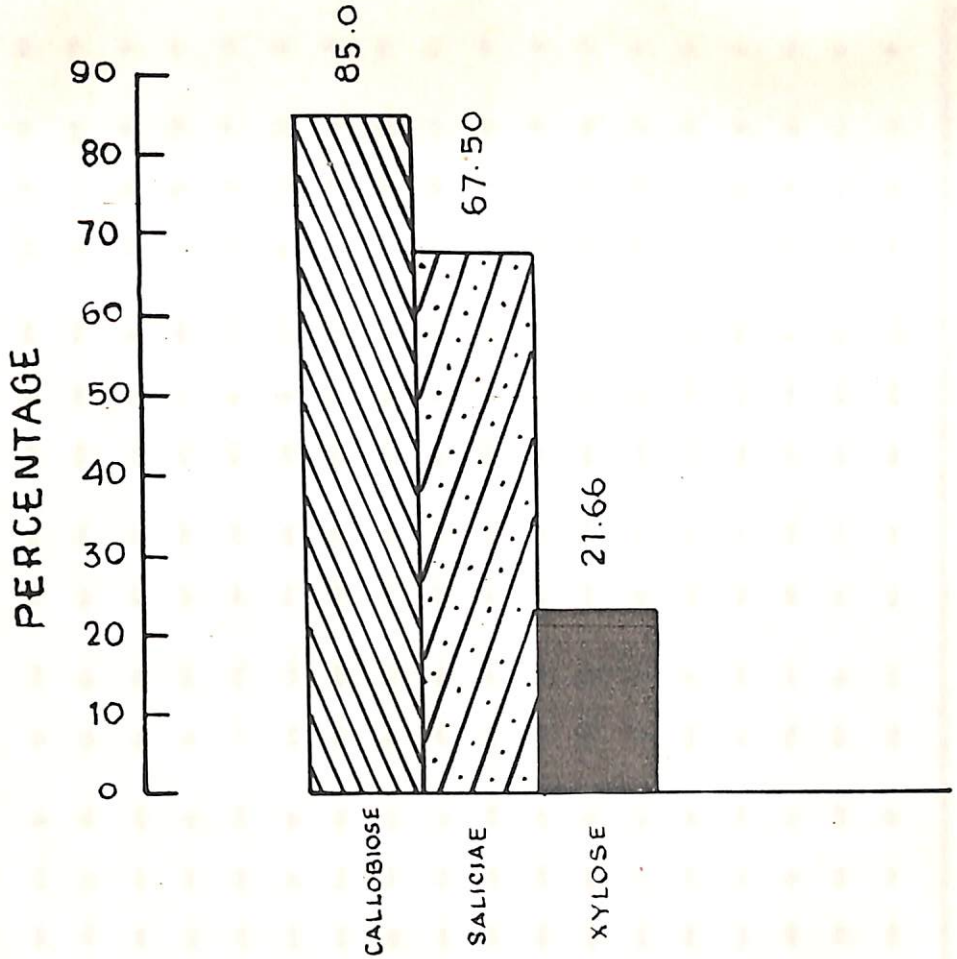

FIG. 7 HISTOGRAM SHOWING FERMENTATION WITH SUGARS

Table 20. Fermentation reaction of Bacillus cereus isolated from chawmin.

Isolate No.	Sugars used for fermentation reaction																
	Glucose	D-Fructose	Sucrose	L-Rhamnose	Mannose	Cellobiose	G-Galactose	Lactose	Mannitol	Xylose	Arabinose	Salicin	D-Sorbitol	Meso-inositol	Glycogen	Dulcitol	Melibiose
1	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
5	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+
12	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	+
14	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
23	+	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+
41	+	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+
49	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	+
50	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+
51	+	+	+	-	-	+	-	-	-	-	-	-	-	-	+	-	+
55	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
56	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-	+
58	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
62	+	+	+	-	-	+	-	-	-	-	-	+	+	-	+	-	+
65	+	+	+	-	-	+	-	-	-	-	-	-	-	-	+	-	+
66	+	+	+	-	-	+	-	-	-	-	+	+	-	-	+	-	+
69	+	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+
74	+	+	+	+	-	-	-	-	-	+	+	-	+	-	-	-	+
75	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+
76	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-	+
78	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	+
79	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+
81	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	+
86	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+

Contd. ...//-

Table 20. Contd. .../-

Isolate No.	Sugars used for fermentation reaction																							
	Glucose	D-Fructose	Sucrose	L-Rhamnose	Mannose	Cellobiose	G-Galactose	Lactose	Mannitol	Xylose	Arabinose	Salicin	D-Sorbitol	Meso-inositol	Glycogen	Dulcitol	Melibiose							
88	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	+							
93	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	+							
94	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	+							
95	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	-	+							
113	+	+	+	+	-	+	-	-	-	-	-	-	+	-	+	-	+							
121	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+							
124	+	+	+	+	-	+	-	-	-	+	+	-	-	-	+	-	+							
126	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-	+							
127	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	+							
132	+	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+							
136	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+							
138	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	-	+							
140	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-	+							
142	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+							
149	+	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-	+							
151	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+							
152	+	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	+							
154	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+							
158	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	+							
160	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+							
167	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+							
168	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+							

Table 21. Fermentation reaction of Bacillus cereus isolated from fried rice.

Isolate No.	Sugars used for fermentation reaction																
	Glucose	D-Fructose	Sucrose	L-Rhamnose	Mannose	Cellobiose	G-Galactose	Lactose	Mannitol	Xylose	Arabinose	Salicin	D-Sorbitol	Meso-inositol	Glycogen	Dulcitol	Melibiose
2	+	+	+	-	-	+	-	-	-	+	-	-	-	-	+	-	+
4	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+
8	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
10	+	+	+	-	-	+	-	-	-	+	-	+	-	-	+	-	+
11	+	+	+	+	-	+	-	-	-	+	-	+	-	-	-	-	+
16	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	+
21	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
22	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
24	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
26	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
32	+	+	+	-	-	+	-	-	-	+	-	+	-	-	-	-	+
40	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-	+
45	+	+	-	-	-	+	-	-	-	+	+	+	-	-	-	-	+
46	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-	+
52	+	+	+	-	-	+	-	-	-	+	-	+	-	-	-	-	+
57	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	+
59	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	+
60	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	+
63	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
64	+	+	+	+	-	+	-	-	-	-	+	-	+	-	-	-	+
70	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	+
71	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
72	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+
73	+	+	+	+	-	+	-	-	-	+	+	-	-	-	-	-	+
77	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
82	+	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	+

Contd. .../-

Table 21. Contd. .../-

Isolate No.	Sugars used for fermentation reaction																
	Glucose	D-Fructose	Sucrose	L-Rhamnose	Mannose	Cellobiose	G-Galactose	Lactose	Mannitol	Xylose	Arabinose	Salicin	D-Sorbitol	Meso-inositol	Glycogen	Dulcitol	Melibiose
83	+	+	+	+	-	+	-	-	-	+	-	-	-	-	+	-	+
87	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	+
92	+	+	+	+	-	+	-	-	-	+	-	-	-	-	+	-	+
96	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
99	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
100	+	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	+
101	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	+
103	+	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	+
104	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	+
105	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
107	+	+	+	+	-	+	-	-	-	-	-	-	+	-	+	-	+
109	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
112	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+
114	+	+	+	-	-	+	-	-	-	-	+	+	+	-	+	-	+
116	+	+	+	+	-	+	-	-	-	-	+	-	-	-	+	-	+
120	+	+	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+
123	+	+	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+
125	+	+	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+
129	+	+	+	+	-	+	-	-	-	-	+	-	+	-	+	-	+
130	+	+	+	-	-	+	-	-	-	-	+	-	-	-	+	-	+
133	+	+	+	-	-	+	-	-	-	-	+	-	-	-	+	-	+
137	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-	+
139	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-	+
141	+	+	+	-	-	+	-	-	-	+	+	+	-	-	+	-	+
143	+	+	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-
148	+	+	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-
150	+	+	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-
155	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+
166	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	+
169	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+

Table 22. Fermentation reaction of Bacillus cereus isolated from skimmed milk powder.

Isolate No.	Sugars used for fermentation reaction																
	Glucose	D-Fructose	Sucrose	L-Rhamnose	Mannose	Cellobiose	G-Galactose	Lactose	Mannitol	Xylose	Arabinose	Salicin	D-Sorbitol	Meso-inositol	Glycogen	Dulcitol	Melibiose
7	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
15	+	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+
19	+	+	+	-	-	+	-	-	-	+	-	+	-	-	-	-	+
25	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
29	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
31	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+
37	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+
38	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
43	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	-	+
44	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
53	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+
54	+	+	+	-	-	-	-	-	-	-	-	+	+	-	+	-	+
80	+	+	+	+	-	-	-	-	-	+	-	+	-	-	+	-	+
84	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	+
85	+	+	+	+	-	-	-	-	-	+	-	-	+	-	+	-	+
89	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-	+
90	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
156	+	+	+	+	-	+	-	-	-	-	-	+	+	-	-	-	+
157	+	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	+

these isolates could be considered as fermenting melibiose. Similarly, these isolates were considered variable (20 - 80 % positive) with respect to fermentation of rhamnose, D-sorbitol, arabinose, xylose, salicin and glycogen. Comparison of these characters of the isolates with those adopted by PHLS,Colindale to identify an isolate as B. cereus is shown in Table 23.

On the basis of fermentation of xylose, salicin and cellobiose these isolates were grouped into different fermentative groups (Table 26), which were considered as biotypes. These were found to be distributed into 8 out of 9 theoretically possible biotypes. The distribution of these isolates according to biotypes is presented in Table 24. The percentage of typability of different biotypes and their distribution on the basis of source of isolates are also presented in the Table 25. Majority of the isolates of fried rice (51.78 %) and chawmin (51.11 %) fell in biotype 7. Out of the 56 isolates of fried rice 29(51.78 %), 15(26.78 %), 9(16.07 %) and 3(5.35 %) fell in biotypes 7, 6, 5 and 2 respectively (Figure 8).

Out of 45 isolates of chawmin 23(51.11 %), 9(20.0 %), 4(8.88 %), 3(6.66 %), 2(4.44) and 1(2.22 %) fell in biotypes 7, 6, 2, 3, 4, 5 and 1 respectively. Two isolates (4.44 %) fell in biotype 8.

All the 19 isolates from skimmed milk powder were biotyped. They fell in biotypes 3(26.31 %), 7(26.31 %) and

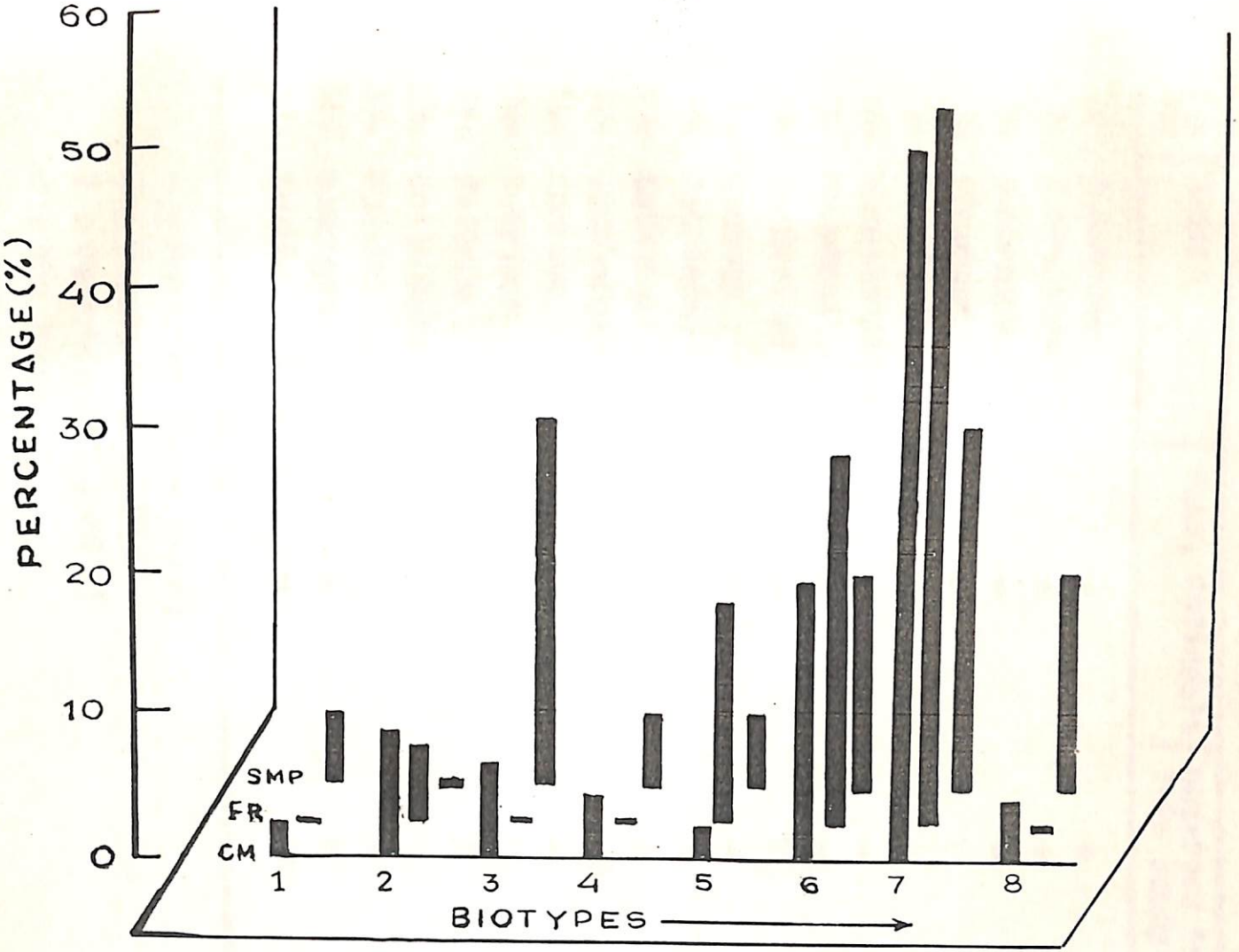

FIG. 8 PERCENT DISTRIBUTION OF BIOTYPES OF B. Cereus ISOLATED FROM CHAWMIN (CM) FRIED RICE (FR) AND SKIMMED MILK POWDER (SMP)

Table 23. Comparison of fermentative reaction of *B. cereus* observed in this study with those reported by PHLS, Colindale.

Sl. No.	Sugars	PHLS, Colindale	Observed in this study	Percent positive
1.	D-Glucose	+	+	
2.	D-Fructose	+	+	
3.	D-Mannose	-	-	
4.	Rhamnose	-	V	50.00
5.	Dulcitol	-	-	
6.	Meso-inositol	-	-	
7.	Mannitol	-	-	
8.	D-Sorbitol	-	V	23.60
9.	Lactose	-	-	
10.	Arabinose	-	V	31.60
11.	Galactose	-	-	
12.	Saccharose	-	ND	
13.	Sucrose	ND	-	
14.	D-Xylose	-	V	21.66
15.	Salicin	+	V	67.50
16.	Cellobiose	+	+	
17.	Melibiose	-	+	
18.	Glycogen	+	V	44.16
19.	Inulin	-	ND	

+ = Positive (80 - 100 %)
- = Negative (0 - 20 %)
V = Variable (20 - 80 %)
ND = Not done.

Table 24. Distribution of *B. cereus* isolates according to biotypes.

Biotypes	Chawmin (n = 45)	Fried rice (n = 56)	Skimmed milk powder (n=19)	Total
1	1 (2.22)	-	1 (5.26)	2
2	4 (8.88)	3 (5.35)	-	7
3	3 (6.66)	-	5 (26.31)	8
4	2 (4.44)	-	1 (5.26)	3
5	1 (2.22)	9 (16.07)	1 (5.26)	11
6	9 (20.00)	15 (26.78)	3 (15.78)	27
7	23 (51.11)	29 (51.78)	5 (26.31)	57
8	2 (4.44)	-	3 (15.78)	5
Total	45	56	19	120

Figures in parenthesis denote the percentages.

Table 25. Distribution of biotypes of *B. cereus* on the basis of source of isolation

Sl. No.	Source of isolates	Biotypes								Total isolates
		1	2	3	4	5	6	7	8	
1.	Chaumin	1/45	4/45	3/45	2/45	1/45	9/45	23/45	2/45	45
2.	Fried rice	-	3/56	-	-	9/56	15/56	29/56	-	56
3.	Skimmed milk powder	1/19	-	5/19	1/19	1/19	3/19	5/19	3/19	19
Percentage typability		1.66	5.83	6.66	2.50	9.16	22.50	57.50	4.16	100.00

Table 26. Biotypes of 120 test isolates of *B. cereus*.

Biotypes	Isolates number	Xylose	Salicin	Cello-biose	Total isolates
1	14,38	-	-	-	2
2	2,16,81,93, 124,168	+	-	+	7
3	1,12,25,29,44, 54,89,95	-	+	-	8
4	5,74,85	+	-	-	3
5	10,11,17,32,40, 45,46,52,141, 143,167	+	+	+	11
6	31,51,64,65,69, 71,72,73,76,83, 84,87,88,90,94, 96,104,107,113, 116,129,130,132, 133,149,150,169	-	-	+	27
7	4,7,8,21,22,24, 26,37,49,50,53, 55,56,57,58,59, 60,62,63,66,70, 75,76,77,79,82, 86,93,100,101,103, 105,109,112,114, 120,121,123,125,126, 136,137,138,139,140, 142,148,151,152,154, 155,156,157,158,160, 166	-	+	+	57
8	15,23,41,43,80	+	+	-	5
Total positive (%)		26 (21.66)	81 (67.50)	102 (85.00)	120

6(15.78 %). Of the remaining three one each was grouped in biotypes 1, 4 and 5. None of the isolates of skimmed milk powder fell in biotype 2.

Serotyping of *B. cereus* isolates with flagellar antigens :

Serotyping was based on the isolates giving agglutination reaction with only of the 20 antisera used and in the event of cross reactions, the antiserum giving highest agglutination titre. In order to rule out cross reactivity, a titre as high as 1:640 was ignored.

The number of isolates agglutinated with single antiserum were 10 (Table 27), 12 (Table 28) and 5 (Table 29) respectively, from chawmin, fried rice and skimmed milk powder. The results of titration of the antisera reacting with more than one and typed on the basis of highest titre antigens of the isolates of *B. cereus* has been presented in Tables 30, 31 and 32 respectively.

Nine isolates from chawmin remained untyped with the 20 antisera used. These were isolates numbers 62, 65, 74, 138, 140, 142, 152, 160 and 167 (Appendix I).

Maximum number of isolates i.e. 6 belonged to serotype No. 5. The details of agglutination test results with all the isolates grouped according to these serotyped and untyped are given in Appendix I. Forty seven isolates from fried rice were serotyped. The frequency distribution of these 47 isolates

Table 27. Agglutination reactions between the H-antigen of such isolates of *B. cereus* that agglutinated with single antisera (chawmin).

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
12	20	40	5120	-	20	-	-	20	20	80	640	40	20	640	320	640	-	20	-	-
23	160	160	1280	80	-	40	640	40	640	80	320	-	-	20	40	640	80	160	20	80
51	-	-	-	-	160	640	-	320	-	640	-	80	80	-	80	-	1280	-	80	80
66	80	80	160	40	40	80	80	80	40	80	160	80	160	80	1280	640	40	20	40	40
76	160	80	-	80	160	80	320	80	320	40	640	320	160	640	5120	320	40	640	640	640
86	-	-	-	-	-	20	80	40	160	80	160	160	-	20	10240	-	40	20	20	-
124	-	20	-	-	20	-	-	-	640	160	20	80	40	-	1280	40	-	-	-	-
127	320	160	320	80	160	640	160	320	160	320	320	640	80	160	80	1280	160	20	160	160
154	20	20	640	40	20	20	20	20	40	20	80	160	-	20	640	1280	-	160	20	20
153	160	20	40	320	1280	320	640	640	640	160	-	80	80	40	320	80	40	20	40	40

Table 28. Agglutination reactions between the H-antigen of such isolates of *B. cereus* that agglutinated with single antisera (fried rice).

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
8	40	-	640	-	-	20	-	640	80	80	-	-	-	20	2560	160	-	20	20	-
11	-	-	640	-	-	-	-	20	-	20	20	-	-	-	1280	80	40	-	-	-
57	-	-	640	-	-	-	-	-	-	20	40	-	-	-	-	2560	-	-	-	-
73	-	20	-	320	10240	40	-	80	40	-	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1280	-	-	-	-	-
112	40	-	640	40	80	80	80	80	40	40	20	80	20	40	320	1280	40	40	40	40
114	-	80	160	20	160	20	320	80	-	160	-	20	20	5120	640	-	-	40	160	80
125	20	20	1280	160	40	40	-	40	-	40	80	20	-	-	-	320	-	80	80	80
129	80	20	2560	20	80	640	640	80	640	20	20	20	40	40	20	-	40	20	320	160
133	[illegible]		160	320	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]			160	80	-	-	80	160	20	20
143	80	40	-	160	320	160	80	40	640	320	80	1280	-	80	-	640	320	160	320	160
169	-	-	-	40	80	-	-	30	320	20	-	20	-	-	1280	640	-	-	-	-

Table 29. Agglutination reactions between the H-antigen of such isolates of *B. cereus* that agglutinated with single antisera (skimmed milk powder).

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FH	HSP_2	RE_2
15	20	-	-	160	40	40	1280	-	-	-	-	-	-	20	160	640	-	20	-	20
17	-	-	2560	-	-	-	-	-	-	-	-	-	-	-	640	20	-	-	40	320
31	20	320	640	320	160	160	80	-	40	40	40	80	20	20	1280	40	-	-	20	20
54	20	40	-	40	80	320	80	640	80	320	1280	640	40	40	80	40	20	80	40	80
90	40	80	-	40	80	-	-	20	20	40	320	640	80	1280	40	-	-	160	80	160

Table 30. Agglutination reactions between the H-antigen of such isolates of B. cereus that agglutinated with more than one antisera (chawmin).

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
1	-	-	1280	-	20	20	-	-	-	-	-	-	-	20	-	2560	-	-	-	-
5	40	-	5120	-	-	20	40	-	-	40	80	640	160	1280	-	40	160	20	-	20
14	20	640	1280	20	1280	640	1280	-	1280	1280	2560	10240	40	-	1280	5120	160	-	-	40
41	640	1280	-	2560	160	-	5120	80	640	160	160	640	640	640	-	2560	-	80	320	80
49	1280	1280	1280	1280	5120	40	640	160	320	40	320	80	320	320	-	2560	40	40	40	40
50	160	320	1280	40	5120	80	10240	320	160	1280	640	5120	160	640	5120	80	80	40	80	80
55	-	-	1280	-	-	-	320	20	-	-	640	-	20	40	2560	2560	640	5120	320	640
56	40	40	2560	80	80	80	40	80	160	160	320	320	80	320	160	640	80	1280	320	320
58	40	20	2560	80	20	40	20	20	40	20	40	40	40	40	160	80	1280	40	20	20
69	320	80	2560	20	20	80	160	80	40	40	40	40	40	40	1280	-	20	20	40	20
75	80	-	40	1280	40	-	80	160	160	1280	20	80	-	-	-	5120	20	-	20	320

Contd. ...../-

Table 30. Contd. ..../-

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
78	80	2560	2560	160	80	40	160	320	2560	5120	640	2560	80	1280	1280	2560	2560	-	80	-
81	640	5120	-	320	320	640	80	1280	5120	1280	2560	-	640	320	10240	-	1280	160	80	1280
88	-	-	-	320	-	-	320	160	2560	1280	1280	-	80	160	160	1280	80	-	-	160
93	640	80	1280	160	80	1280	320	640	640	320	320	640	320	2560	160	1280	640	320	640	320
94	640	2560	160	320	160	640	640	5120	1280	640	1280	2560	320	2560	320	320	320	320	2560	640
95	320	640	1280	80	160	320	160	640	5120	2560	2560	2560	320	40	320	2560	1280	640	640	1280
121	160	80	640	320	40	40	20	2560	5120	2560	2560	640	1280	640	40	320	80	160	640	2560
126	2560	1280	80	-	20	160	320	320	5120	640	160	1280	160	640	20	20	320	320	2560	160
149	160	40	-	160	80	320	2560	20	320	2560	2560	10240	160	1280	2560	5120	640	1280	640	2560
151	640	80	640	40	160	40	160	-	320	320	640	2560	10240	-	1280	2560	320	1280	320	320
168	160	80	5120	80	80	80	160	320	320	1280	2560	640	320	20	10240	1280	80	20	320	320

Table 31. Agglutination reactions between the H-antigen of such isolates of *B. cereus* that agglutinated with more than one antisera (fried rice).

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	3	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
2	-	20	80	1280	40	640	160	80	40	-	320	5120	-	-	2560	640	20	20	20	20
4	-	-	1280	-	-	40	-	-	-	40	20	-	-	-	640	5120	-	20	-	80
10	20	40	40	160	20	320	1280	80	5120	20	1280	40	80	40	160	320	20	320	640	1280
16	-	-	640	-	20	-	-	20	-	-	-	-	-	20	1280	2560	-	-	-	-
21	-	-	5120	-	20	-	-	20	-	20	-	-	-	20	1280	1280	-	-	-	-
22	-	2560	2560		1280	-	640	-	10240	-	40	-	-	-	-	1280	-	-	-	-
24	20480	-	1280	-	40	-	-	40	-	-	-	-	-	20	2560	-	5120	5120	5120	-
26	160	20	80	320	-	-	640	160	320	1280	-	320	20	160	10240	320	20	80	80	5120
32	-	80	1280	160	40	320	320	80	160	80	160	-	20	320	40	5120	40	640	20	-
40	-	-	10240	-	-	-	-	-	-	-	-	-	-	-	2560	2560	-	-	-	-
45	-	20480	-	640	10240	40	2560	80	40	40	-	20	-	10240	-	160	320	80	-	20
46	20	-	160	-	-	-	80	-	80	80	-	20480	20	-	5120	1280	-	-	-	-
52	-	20	2560	-	10240	20	-	-	80	-	-	-	-	-	5120	1280	-	-	-	-
59	20480	-	[illegible]	-	[illegible]	[illegible]			[illegible]	20	-	-	-	20	-	-	10240	-	-	5120
60	5120	-	-	-	-	1280	-	-	-	20	-	-	-	20	160	2560	10240	-	2560	-
63	1280	20	-	-	-	640	-	640	-	640	640	-	-	40	40	320	2560	-	-	-
64	-	-	40	-	-	-	-	-	-	20480	160	-	5120	-	80	80	-	-	-	-
70	-	-	2560	-	-	20	-	-	-	-	40	-	-	40	320	10240	-	-	20	-

Contd. .../-

Table 31. Contd. ...7-

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
71	-	20	2560	-	-	-	40	-	-	-	-	-	-	-	10240	-	-	-	-	-
72	640	160	320	320	80	-	1280	20480	2560	40	-	-	2560	2560	-	1280	640	-	80	80
82	-	-	1280	-	-	-	-	-	80	80	-	-	-	-	5120	80	-	-	80	20
87	-	-	5120	-	-	-	-	-	-	20	-	20	-	40	1280	2560	-	-	-	-
92	-	640	2560	640	640	80	-	1280	20480	320	80	20	80	80	5120	320	80	-	1280	1280
96	-	-	640	-	40	5120	20	40	20	-	2560	20	160	80	-	1280	80	40	640	40
99	-	160	1280	640	-	1280	-	640	2560	640	-	-	80	-	1280	320	-	5120	5120	10240
101	80	640	2560	160	80	320	-	20	20	80	5120	20	40	40	160	-	40	80	-	-
107	80	160	1280	80	80	80	80	80	160	320	40	80	40	640	-	80	2560	80	80	40
116	20	160	20	320	320	640	2560	320	640	320	640	20	320	160	-	320	320	1280	40	-
120	80	40	80	80	80	80	40	80	1280	640	320	5120	320	2560	160	-	640	320	160	320
130	5120	1280	2560	640	2560	320	20	320	320	640	160	640	2560	640	40	80	160	640	320	40
139	1280	40	-	640	40	80	10240	1280	1280	5120	320	320	5120	5120	1280	-	40	40	640	160
141	-	-	-	80	-	-	40	160	80	320	640	80	2560	1280	-	1280	640	640	160	20
148	1280	640	20	320	640	1280	160	160	640	1280	20	1280	20	40	-	20	5120	40	160	320
150	[illegible]	320	[illegible]	-			640	40	160	160	1280	10240	640	40	-	20	40	160	40	40
155	160	40	80	80	80	40	20	80	20	80	20	40	-	80	2560	1280	40	40	160	160

Table 32. Agglutination reactions between the H-antigen of such isolates of *B. cereus* that agglutinated with more than one antisera (skimmed milk powder).

Antigen No.	Agglutination titre with antisera (serotype)																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HCP_2	RE_2
25	-	-	2560	-	-	-	-	-	-	-	20	-	-	-	5120	1280	-	-	-	-
29	20	80	2560	640	20	320	-	-	160	20	40	80	40	20	-	1280	320	20	-	-
37	160	80	1280	1280	160	1280	80	320	160	160	320	320	20	20	5120	-	40	-	-	-
43	20	20	80	20	40	20	20	20	80	20	640	320	20	20	2560	5120	-	-	-	20
44	40	80	1280	640	40	40	160	320	640	80	320	160	80	80	2560	160	80	160	320	640
84	20	40	160	320	80	5120	2560	2560	80	2560	80	1280	40	2560	320	1280	2560	20	20	20
85	20	80	160	320	20	40	640	160	80	40	320	640	80	160	1280	2560	320	80	2560	5120
156	40	160	1280	320	160	1280	80	1280	40	20	640	320	160	40	2560	20	1280	1280	80	-
157	40	20	160	80	320	2560	320	320	160	1280	1280	1280	80	5120	20	-	2560	1280	-	-

is given in Table 34. Twelve isolates agglutinated with only one antiserum. Five isolates of the fried rice did not agglutinate with any of the 20 antisera tested in a titre $\geq$ 640. In the case of another 4 isolates it was not possible to decide any serotype because these cross reacted with more than one antiserum in titre $\geq$ 640. Therefore, a total of nine isolates were considered as untyped. The details of the agglutination test and titres of all the isolates from fried rice are summarised up in Appendix II.

Out of 19 isolates of skimmed milk powder tested 14 could be serotyped against the antisera tested. Majority of the isolates fell in serotype 26(2), followed by 24(2) and 5(2), serotypes 8, 11, 17, RE_2 and 75 one each. Two isolates did not react with any antisera tested. These isolates were 7 and 38. Five isolates reacted with only one sera. Isolates bearing number 53, 80 and 89 showed cross reactions with more than one antisera with equal titre and hence, could not be serotyped (Appendix III).

The description of all 120 isolates with respect to their origin, serotype and biotype has been presented in Tables 35, 36 and 37. Altogether 93 out of 120 isolates could be serotyped while all the isolates were biotyped. Summary of the results of serotyping is presented in Table 33. For the delineation of the isolates both serotyping and biotyping should be done.

Table 33. Summary of results of serotyping of isolates of *B. cereus*.

Sl.	Source	Chawmin	Fried rice	Skimmed milk powder	Total
1.	Total number tested	45	56	19	120
2.	Serotyped	32 (71.11)	47 (83.92)	14 (77.77)	93
	a) Serotyped on the basis of agglutination with single serum	10 (22.22)	12 (21.42)	5 (26.31)	27
	b) Serotyped on the basis of highest titre with single serum	22 (48.88)	35 (62.50)	9 (47.36)	66
3.	Totally untyped	13 (28.88)	9 (16.07)	5 (26.31)	27

Figures in parenthesis indicates the percentages.

Table 34. Frequency distribution of serotypes of the isolates from chawmin, fried rice and skimmed milk powder.

Serotypes	Isolates from		
	Chawmin	Fried rice	Skimmed milk powder
1	-	3/47	-
2	-	1/47	-
5	6/32	4/47	2/14
6	-	-	-
7	2/32	2/47	-
8	-	1/47	1/14
11	2/32	2/47	1/14
13	1/32	2/47	-
14	5/32	2/47	-
15	1/32	1/47	-
17	-	1/47	1/14
18	2/32	6/47	-
21	1/32	1/47	-
24	1/32	1/47	2/14
26	5/32	9/47	5/14
75	4/32	6/47	1/14
Respa	1/32	4/47	-
FM	1/32	-	-
HSP_2	-	-	-
RE_2	-	1/47	1/14

Table 35. Serotype and biotype of *B. cereus* isolated from chawmin.

Sl.No.	Isolate No.	Serotype	Biotype
1	1	75	3
2	5	5	4
3	12	5	3
4	14	18	1
5	23	5	8
6	41	11	
7	49	7	7
8	50	11	7
9	51	Respa	6
10	55	FM	7
11	56	5	7
12	58	5	7
13	62	NT	7
14	65	NT	6
15	66	26	7
16	69	5	6
17	74	NT	4
18	75	75	7
19	76	26	6
20	78	15	7
21	79	NT	7
22	81	14	2
23	86	26	7
24	88	14	6
25	93	24	2
26	94	13	6
27	95	14	3
28	113	NT	6
29	121	14	7
30	124	26	2
31	126	14	7
32	127	75	7
33	132	NT	6
34	136	NT	7
35	138	NT	7
36	140	NT	7
37	142	NT	7
38	149	18	6
39	151	21	7
40	152	NT	7
41	154	75	7
42	158	7	7
43	160	NT	7
44	167	NT	5
45	168	26	2

NT = Note typed

Table 36. Serotype and biotype of *B. cereus* isolated from fried rice.

Sl.No.	Isolate No.	Serotype	Biotype
1	2	18	2
2	4	75	7
3	8	26	7
4	10	14	5
5	11	26	5
6	13	26	7
7	16	75	2
8	21	5	7
9	22	13	7
10	24	1	7
11	32	75	5
12	40	5	5
13	45	2	5
14	46	18	5
15	52	7	5
16	57	75	7
17	59	1	7
18	60	Respa	7
19	63	Respa	7
20	64	15	6
21	70	75	7
22	71	26	6
23	72	13	6
24	73	7	6
25	77	26	7
26	82	26	7
27	83	NT	6
28	87	5	6

Contd. ...

Table 36. Contd. .../-

Sl.No.	Isolate No.	Serotype	Biotype
29	92	14	2
30	96	8	6
31	99	RE_2	7
32	100	NT	7
33	101	17	7
34	103	NT	7
35	104	NT	6
36	105	NT	7
37	107	Respa	6
38	109	NT	7
39	112	75	7
40	114	24	7
41	116	11	6
42	120	18	7
43	123	NT	7
44	125	5	7
45	129	26	6
46	130	1	6
47	133	18	6
48	137	NT	7
49	139	11	7
50	141	21	5
51	143	18	5
52	148	Respa	7
53	150	18	6
54	155	26	7
55	166	NT	7
56	169	26	6

NT = Not typed

Table 37. Serotype and biotype of *B. cereus* isolated from skimmed milk powder.

Sl.No.	Isolate No.	Serotype	Biotype
1	7	NT	7
2	15	11	8
3	17	5	5
4	25	26	3
5	29	5	3
6	31	26	6
7	37	26	7
8	38	NT	1
9	43	75	8
10	44	26	3
11	53	NT	7
12	54	17	3
13	80	NT	8
14	84	8	6
15	85	RE_2	4
16	89	NT	3
17	90	24	6
18	156	26	7
19	157	24	7

NT = Not typed

DISCUSSION

Bacillus cereus has recently emerged as one of the most important food borne pathogen in India. It is most commonly associated with cereal foods causing diarrhoea and vomition in human beings. As compared to other food borne pathogens, relatively little work has been done on *B. cereus* particularly with 'fast foods'.

In recent years, the consumption of ready to eat foods like chawmin and fried rice from fast food centre and Chinese style hotels/ restaurants has increased. Customers of fast food restaurants have increased and on the other hand the time to prepare a dish/ food and serve is short. In other words the processing of a 'fast foods' is often inadequate to decontaminate or considerably reduce the number of bacteria in the food. As a result, there is a likelihood of food poisoning affecting large number of people. Therefore, the present investigation was undertaken to see the incidence of this organism in chawmin and fried rice. Skimmed milk powder was also included in this study.

Altogether 23.0 per cent samples of chawmin were contaminated with *B. cereus* and the counts ranged from zero to 3.9193 with an average of 0.6453 $\pm$ 0.1225/g. Similarly 24.0 per cent of fried rice samples were contaminated with *B. cereus* compared to 100.0 per cent in boild rice samples observed by Kamat *et al.* (1989). The levels of contamination of fried rice observed by Gilbert (1979) and Glenn *et al.* (1988) were higher

than those observed in this study. However, in boiled rice, the level of contamination observed by Kamat et al. (1989) was more or less similar to those observed in this study. In general the results of this study indicated that (a) smaller samples of fried rice were contaminated and (b) the level of contamination was low. No significant difference was noted in the level of contamination, range and per cent positivity for B. cereus between chawmin and fried rice samples.

Informations about contamination of chawmin as was observed in this study appears to be the first.

A low level of contamination observed during the present study may be because of several reasons. Quantitative accumulation of B. cereus in a food depends upon the number of organisms initially present, the suitability of the food as growth medium and the length of time, the food is left at optimum temperature for growth. Harmon and Kautter (1991) reported a count of 7.9×10^6/g of rice and 6.7×10^6/g of noodles 20 hours after incubation. In this study samples were collected just after frying. The common practice is to serve the food to the consumers just after frying. Since aerobic spore forming bacteria like B. cereus can survive moderate heat, spores surviving in cooked food may germinate and multiply when the food is held for longer period at favourable/ ambient temperature. This long time of holding was not allowed in this study and therefore low level of B. cereus count was possibly observed in contrast to those reported by Harmon and Kautter (1991) cited above. It is to be

mentioned that most of the samples of fried rice except one showed a count of ≥ 5.0/g which is considered as minimum dose for the onset of B. cereus food poisoning (Kramer et al., 1982). All 23 samples of chawmin positive for B. cereus had a count of ≥ 2.0 to ≥ 4.0. Thirteen samples had counts ≥ 3.0. Similarly 21/24 samples of fried rice had counts between ≥ 2.0 to ≥ 4.0 and 11/24 had ≥ 3.0. These samples viz. 13/23 of chawmin and 11/24 of fried rice met the guide line/ standard of New Hampshire, Department of Health & Welfare, USA which suggested 5×10^3 B. cereus /g of non-packaged foods, general (Wehr, 1982). The practice of selling foods just after frying to the consumers appears to be healthy. If the time gap between preparation and consumption exceeds, there is every chance of contracting food borne infection with B. cereus through these foods.

Altogether 69.23 per cent samples of skimmed milk powder were found contaminated with B. cereus. The level of B. cereus contamination in skimmed milk powder ranged from zero to 3.6812/g with an average of 1.9386 ± 0.2736/g. The results obtained in this study are in agreement with those of previous studies related to the wide spread B. cereus contamination of milk products (Ahmed et al., 1983; Hin-Chung et al., 1988). Contamination of dairy products with B. cereus may be traced back to the environment or mastitic bovine sources (Davies and Wilkinson, 1973).

A high incidence of contamination of skimmed milk powder with B. cereus might be due to :

(a) Initial higher contamination of raw milk with B. cereus.

(b) Heat processing not sufficient to destroy the spore forming organisms.

(c) Heat shock activates spores to germinate in vegetative form.

(d) Milk powder is a favourable medium for growth and multiplication of the organisms.

The difference between percentage positivity for B. cereus in samples of chawmin and fried rice was non significant (52.0 % and 60.0 % respectively) while significantly more samples of skimmed milk powder (69.23 %) were found contaminated.

No significant difference in level of contamination, percentage positivity and range of bacterial contamination with B. cereus was observed in samples of chawmin and fried rice. It may be because both are of cereal origin and were obtained from the same fast food restaurant, the hygienic standards of which were common to both the foods.

In this study the medium used for direct plating was MEYPA. This being inbibitory to many bacteria only limited group of organism grew on it. The number of such bacteria was also estimated. This was designated as "bacteria other than B. cereus". Such bacteria include S. aureus, Serratia marcescens and P. vulgaris (Holbrook and Anderson, 1980).

Although no attempt was made to isolate and identify these, their detection and enumeration indicated that the food was certainly contaminated. As many as 74.0 per cent and 64.0 per cent of chawmin and fried rice respectively were found contaminated with bacteria other than B. cereus. This again suggested that the majority of the foods served i.e. chawmin and fried rice were free from B. cereus. Since, bacteria growing on MEYPA plate include the pathogen like S. aureus, although specific identification was not done, consumption of such foods could be associated with the risk of food poisoning.

The counts of bacteria other than B. cereus ranged from zero to 5.4771 with an average of 2.8606 ± 0.1852/g. In case of fried rice counts ranged from zero to 5.6989 with an average of 2.5543 ± 0.1990/g. According to Ohio, Department of Agriculture, USA, in macaroni salads and precooked ready to eat foods a count of 1×10^3 S. aureus could be permitted (Wehr, 1982). Even if it was considered that S. aureus alone (although no specific identification was done) contributed to the average count of 2.8606 ± 0.1852 these food samples were satisfactory. Whether B. cereus or S. aureus was considered bacteriological parameter, the quality of chawmin and fried rice was thus satisfactory.

While working in a Food Hygiene Laboratory, it is often desired to estimate the count of specific pathogen /g of food. However, due to lack of suitable selective and differential medium one has to be satisfied with estimating

presumptive count of a pathogen. If time permits, further laboratory tests are done in order to confirm. In the case of B. cereus this difficulty was experienced. A presumptive count of B. cereus is based upon mainly the Lecithinovitelline positive mannitol negative as observed on MEYPA. The confirmation requires a number of tests.

Kramer et al. (1982) recommended production of acid in Ammonium salt sugar medium containing glucose and no acid production in arabinose, mannitol and xylose as additional tests for confirmation. In this study Voges-Proskaeur reaction was added because it enabled differentiation of B. cereus from atleast five different species of Bacillus. Since xylose positive isolates were common in India (Deva, 1989; Mondal, 1990; Jha, 1992) contrary to those reported by others (Kramer et al., 1982; Shinagawa, 1990) both positive and negative reactions were considered. B. cereus was thus defined in this study as a Gram positive, motile, facultative anaerobic, endospore forming bacilli, which were catalase positive, VP positive, mannitol negative, glucose positive, arabinose negative, xylose variable. It was possible in this study to make a comparison between the number of samples and per cent of colonies presumptively identified as B. cereus and later on confirmed after detailed studies of characters defined above. In 60.0 to 100.0 per cent instances the samples found to be positive for B. cereus presumptively were also confirmed. Further, 88.0 ± 1.743 per cent of colonies presumptively identified were also confirmed as B. cereus. Such

a quantitative evaluation of the reliability of presumptive identification and count of B. cereus on MEYPA has not been reported earlier.

Bacillus cereus produces two types of enterotoxin. The 'diarrhoeal syndrome' type is similar to those of Clostridium perfringens food poisoning. The emetic syndrome type of B. cereus resembles those seen in staphylococcal enterotoxin intoxication. Food borne illness due to Clostridium perfringens and Staphylococcus aureus were well established earlier. This situation may be responsible for B. cereus being overlooked as a potential causative agent in some of the early outbreaks. Certainly, without laboratory confirmation, it would not be possible to establish B. cereus food poisoning.

A review of biochemical characters used to study B. cereus suggested their inadequancy in identification. The characters described by Wilson and Miles (1975), Gregersen and Halgaard (1976) and Holt (1977) failed to correctly identify Bacillus spp. as Deva and Narayan (1988) considered some of their isolates as B. circulans and B. coagulans on these basis but the same isolates were later identified as B. cereus by PHLS, Colindale. Study of large number of isolates of B. cereus by Gilbert et al. (1981) suggested that an isolate which was motile, formed characteristic colony on blood agar, was lecithino-vitelline positive, fermented glucose but not mannitol, arabinose and xylose was regarded as B. cereus. Non motile strains of B. cereus were also included. A set of fifteen characteristics was required for confirmation. Even these criteria would miss an

isolate of B. cereus particularly if one was attempting to identify a culture not at all connected with food poisoning.

In India, only two reports have been published on the possible association of B. cereus in food poisoning/ gastro-enteritis. Detailed study of the B. cereus isolates from food in India has been made only in this laboratory. Previously 40 strains of B. cereus were isolated in this laboratory. Out of which 26 isolates were not typable by PHLS, Colindale with their serotyping system. Of these 37.5 per cent isolates were xylose positive and phage resistant. These strains had been reported as emetic type enterotoxin producer. Singh (1990), on the contrary, reported that three of these 26 strains produced diarrhoeal enterotoxin.

These illustrated that Indian isolates were different from those of European strains. A detail study of biochemical and serological characters of these isolates was done by Jha (1992). He described a biotyping system and also raised 17 antisera for serotyping. In this study, both biotyping and serotyping were done. Serotyping included antisera raised against additional three isolates from this study.

In this study, all the tests for identification recommended by Gilbert et al. (1981) were included. In addition, tests for fermentation of sugars listed in API 50CH of PHLS, Colindale were also done. Seventeen out of forty sugars were also included in this study. The criteria of declaring a test

positive/ negative/ variable adopted was 80.0 to 100.0 per cent positive, $\leq$ 20.0 per cent negative and 20.0 to 80.0 per cent variable. Of the sugars tested, fermentation of xylose, appeared to be important marker for distinguishing Indian strains from European ones. Gilbert et al. (1981) reported that not even a single strain of B. cereus out of 2,275 tested, was xylose positive. Contrary to this 26 out of 120 (21.66 %) of isolates in this study fermented xylose. The results of this study and as also earlier ones (Deva, 1989; Mondal, 1990; Jha, 1992) established that the Indian isolates were different from those of the European strains of B. cereus.

Gilbert and Taylor (1976) reported that the strains of B. cereus isolated from emetic type of food poisoning cases do not ferment salicin. In this study 81 out of 120 (67.5 %) isolates from fried rice, chaumin, and skimmed milk powder fermented salicin. Earlier study (Jha, 1992) reported that 20 isolates from pork products and ingredients fermented salicin. These isolates were not associated with food poisoning outbreaks. Further, 25 isolates of B. cereus studied by Singh (1990) and Mondal (1990) produced diarrhoeal type enterotoxin. These findings confirmed that salicin fermentation was not indicative of emetic type of enterotoxin production by a strain. Infact, these are two different characters. The strains of B. cereus under study were found to be distributed in 8 out of 9 theoretically possible biotypes.

The isolates exhibited variable reaction with respect to fermentation of xylose, salicin and positive for cellobiose. This variability in fermentation pattern was utilized for biotyping. Jha (1992) found that majority of his isolates fell in biotype 3 while during the present study majority of isolates fell in biotype 7.

Biotyping may be used for epidemiological investigation as it helped in locating the source of an isolate.

During the course of present study the majority of isolates from samples of chawmin fell in biotype 7(51.11 %). Similarly in the case of fried rice majority of the isolates fell in biotype 7(71.78 %) followed by 8(26.78 %) and 9(16.07 %) and biotype 3(5.35 %). Similarity of biotype 7 for majority of the isolates of B. cereus from chawmin and fried rice indicated a common source of contamination which was the fact as both the samples were drawn from the same sources. None of the isolates of fried rice fell in biotypes 1, 3, 4 and 8. Compared to raw rice, chawmin is a processed product. Therefore, there is a possibility of acquiring B. cereus from varied sources during preparation of raw chawmin. Distribution of B. cereus isolated from chawmin in 8 biotypes in contrast to distribution of the isolates from fried rice in only 4 biotypes is thus explained. A comparison of the frequency distribution of isolates of B. cereus originating from chawmin, fried rice, skimmed milk powder on the one hand and from spices and pork products on the

other reported by Jha (1992), suggests that biotyping is an useful tool (Table 38). It appears that biotype 3 was specific for pork products and their ingredients such as spices and flour while biotype 7 was specific for other like chawmin and fried rice.

In case of skimmed milk powder sample majority of isolates were grouped in biotypes 7(26.31 %) and 3(26.31 %). None of the isolates fell in biotype 2.

As reported by Jha (1992) majority of the isolates from flour, spices and pork products (23.80, 50.0 and 30.43 %, respectively) fell in biotype 3. This may be because flour and spices are used for the preparation of the pork products. None of his isolates from flour fell in biotypes 5, 6 and 8. The isolates from flour fell in biotypes 1 and 3 only. None of his isolates from spices fell in biotypes 2, 4, 5, 6, 7 and 8, while majority of isolates obtained during the course of study fell in biotypes 6 and 7. Thus it is obvious that biotyping can delineate isolates as per the source. Therefore it may be used for epidemiological investigation. It may help in locating the source of an isolate.

Another useful tool for epidemiological investigation was serotyping. Previously, the isolates of B. cereus sent from the Department of Veterinary Public Health & Epidemiology, Ranchi Veterinary College, Ranchi to PHLS, Colindale, could not be serotyped, with British system of serotyping. This necessitated, raising antisera against Indian isolates.

Table 38. Ability of biotyping of isolates of *B. cereus* in delineating source of contamination.

Biotypes	Observed during this study			Observed by Jha (1992)		
	Chawmin	Fried rice	Skimmed milk powder	Flour	Spices	Pork Products
1	1(2.22)	-	1(5.26)	2(16.66)	1(25.00)	2(8.69)
2	4(8.88)	3(5.35)	-	1(8.33)	-	5(21.73)
3	3(6.66)	-	5(26.31)	5(23.80)	2(50.00)	7(30.43)
4	2(4.44)	-	1(5.26)	2(16.66)	-	6(26.08)
5	1(2.22)	9(16.07)	1(5.26)	-	-	1(4.34)
6	9(20.00)	15(26.78)	3(15.78)	-	-	1(4.34)
7	23(51.11)	29(51.78)	5(26.31)	2(16.66)	-	1(4.34)
8	2(4.44)	-	3(15.78)	-	-	-

Figures in parenthesis indicates the percentages.

The serotyping with flagellar antigen of these 120 isolates was done. Previously, Kramer et al. (1982) experienced antigenic cross reactivity and therefore decided to ignore agglutination titre of 1:400. Jha (1992) ignored a titre of 1:640 to avoid cross reaction. In the present study also a titre of 1:640 were ignored.

With a battery of 20 antisera it was possible to type 93 out of 120 isolates. Clearly serotyped were the isolates which did not cross react and these were 27 out of 93. Whereas 61 were serotyped on the basis of highest agglutinating titre. Majority of the isolates from chaumin fell in the serotypes 5, 14 and 26 while the isolates from fried rice fell in serotypes 26, 18 and 75. Those of the skimmed milk powder fell in serotype 5. It is interesting that 31 of the 93 serotyped isolates were agglutinated with the three antisera raised from the isolates from chaumin and fried rice. Thus cluster of isolates fell into these three serotypes. This indicated that a battery of antisera to be used for serotyping should include antigens from varied sources. This is also supported by the fact that majority of the isolates from pork products and their ingredients such as flour and spices belonged to different serotypes (Table 39) as was observed by Jha (1992). It may further be concluded that serotyping is advantageous in epidemiological studies, as has been widely used in England and Japan.

Table 39. Ability of serotyping of isolates of *B. cereus* in delineating source of contamination.

Serotypes	Observed during this study			Observed by Jha (1992)		
	Chawmin	Fried rice	Skimmed milk powder	Flour	Spices	Pork products
1	-	3	-	1	-	2
2	-	1	-	1	-	2
5	6	4	2	-	-	-
6	-	-	-	-	-	-
7	2	2	-	-	-	2
8	-	1	1	-	-	-
11	2	2	1	-	-	-
13	1	2	-	-	-	-
14	5	2	-	-	-	-
15	1	1	-	-	-	-
17	-	1	1	-	-	1
18	2	6	-	1	-	-
24	1	1	-	-	1	2
26	5	9	5	-	-	-
75	4	6	1	-	-	-
Respa	1	4	-	-	1	1
FM	1	-	-	1	-	-
HSP_2	-	-	-	2	-	2
RE_2	-	1	1	1	-	5

Comprehensive analysis of the studies made in this laboratory including the present one strongly suggests development of a battery of antisera, which should be mono-specific.

List of isolates which should be used for raising battery of antisera with antigens PV_2, FA, HSP Jha, 1992) and 7, 38, 62, 65, 74, 83, 103, 104, 137, 138, 140, 142, 152, 160, 166 and 167.

For a precise epidemiological studies of isolates from vomitus or stool of a victim of food poisoning and the incriminated food, description of both serotype and biotype is essential. Both the systems are complimentary to each other and not a substitute of either.

SUMMARY & CONCLUSION

Bacillus cereus has recently emerged as one of the most important food borne pathogen in India. It is most commonly associated with cereal foods causing diarrhoea and vomition in human being. As compared to other food borne pathogens relatively little work has been done on *B. cereus* particularly with 'fast foods'. Therefore, the present investigation was undertaken to see the incidence of this organism in chawmin and fried rice. Skimmed milk powder was also included in this study.

Altogether 226 samples consisting of chawmin (100), fried rice (100) and skimmed milk powder (26) were examined. The results has been presented in $\log_{10}$ scale. Altogether 23.0, 24.0 and 69.23 per cent of the samples of chawmin, fried rice and skimmed milk powder were positive for *B. cereus* and the counts ranged between zero to 3.9193/g, zero to 3.6182/g and zero to 3.6812/g with an average of 0.6453 $\pm$ 0.1225, 0.7394 $\pm$ 0.1400 and 1.9386 $\pm$ 0.2736/g, respectively.

Mannitol egg yolk polymixin agar appears to be a satisfactory selective and differential medium for *B. cereus* which is inhibitory to many bacteria and only a limited group of organisms grew on it. Viz. *S. aureus*, *Serratia marcescens* and *P. vulgaris*. The number of such bacteria was also estimated and designated as 'bacteria other than *B. cereus*'. Altogether 74.0, 64.0 and zero per cent of the samples of chawmin, fried rice and skimmed milk powder were positive for bacteria other than *B. cereus* and the count ranged from zero to 5.4771, zero

to 5.6989 and zero respectively. The average counts of bacteria other than B. cereus in above foods were 2.8606 ± 0.1852, 2.5543 ± 0.1990 and zero /g, respectively. No significant difference in level of contamination with B. cereus was observed in chawmin and fried rice, while per cent positivity for B. cereus was significantly higher (69.23 %) in skimmed milk powder than in chawmin and fried rice (52.0 and 60.0 %), respectively.

Difference between percentage positivity for B. cereus in samples of chawmin and fried rice was non significant while significantly more samples of skimmed milk powder were found positive.

Information about contamination of chawmin as observed in this study appears to be the first.

A comparison of the test methods for detection of B. cereus viz. direct plating Vs enrichment was made. Normal deviate test revealed significant difference between positivity for B. cereus by direct plating and enrichment method in chawmin ($P < 0.05$), fried rice ($P < 0.01$) and skimmed milk powder. Per cent positivity by enrichment was significantly higher than by direct plating in chawmin and fried rice. However, opposite trend was observed in skimmed milk powder i.e. higher per cent positivity by direct plating than by enrichment method.

In 60.0 to 100.0 per cent instances the samples were found positive for B. cereus presumptively were also confirmed. Further, 88.0 ± 1.7430 per cent of colonies presumptively

identified were also confirmed as B. cereus. Such a quantitative evaluation of the reliability of presumptive identification and count of B. cereus on MEYPA has not been reported earlier.

This laboratory has earlier experienced that 100.0 per cent isolates of B. cereus of Indian origin were non-typable with British serotyping system. Therefore, suitable markers (biotyping and serotyping) are needed for epidemiological studies.

All the isolates of B. cereus were subjected to 17 different sugar fermentation tests. The isolates were found variable (i.e. 20.0 to 80.0 % of strains) with respect to fermentation of xylose and salicin. The fermentation of xylose was important for the differentiation of Indian strains from British ones. Our isolates (21.66 %) as against less than one per cent of British strains fermented xylose on the basis of biochemical characters, these fermented xylose (21.66 %), salicin (67.50 %) and cellobiose (85.0 %).

On the basis of the fermentation of three sugars viz. xylose, salicin and cellobiose the biotyping was done. All strains under this study were found to be distributed in 8 out of 9 theoretically possible biotypes. It is interesting that all strains were clearly distinguished on the basis of biotypes. Thus it was concluded that biotyping might be useful for delineating the source of an isolates.

It was concluded that biotyping was as good as or better than serotyping in locating the origin of an isolates. Biotyping may prove helpful where strains could not be differentiated on the basis of serotypes. Therefore, for making precise identification of an isolate of B. cereus both serotyping and biotyping was recommended.

Serotyping was based on the isolates giving agglutination reaction with 20 antisera used and in the event of cross reactions, the antisera giving highest agglutination titre. In order to rule out cross reactivity, a titre as high as 1:640 was ignored.

With a battery of 20 antisera it was possible to type 93 out of 120 isolates. Clearly serotyped were the isolates which did not cross react and these were 27 out of 93 whereas 66 were serotyped on the basis of highest agglutinating titre.

Comprehensive analysis of the studies made in this laboratory including the present one strongly suggests development of a battery of antisera which should be monospecific. List of isolates to be used for raising battery of antisera with antigens are PV_2, FA, HSP, 7, 38, 62, 65, 74, 83, 103, 104, 137, 138, 140, 142, 152, 160, 166 and 167.

For a precise epidemiological studies of isolates from vomitus or stool of a victim of food poisoning and the incriminated food, description of both serotype and biotype is essential. Both the systems are complimentary to each other and not a substitute either.

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APPENDICES

APPENDIX

MEDIA AND REAGENTS

1. Ammonia salt sugar (ASS) :

$(NH_4)_2 HPO_4$	1.0 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Yeast extract	0.2 g
Agar	20.0 g
Distilled water	1000 ml
Bromocresol purple (0.2 % solution)	4.0 ml

Add sugar to 1.0 per cent concentration. Autoclave at 115°C for 20 minutes.

2. Gelatin agar :

Gelatin	4.0 g
Distilled water	50 ml
Nutrient agar	950 ml

Soak the gelatin in the water and when thoroughly softened and add to melted nutrient agar.

3. Mannitol-egg-yolk-polymixin (MEYP) agar :

Meat extract	1.0 g
Peptone	10.0 g
D-mannitol	10.0 g
NaCl	10.0 g

Phenol red	0.025 g
Agar	20.0 g
Distilled water	1000 ml
pH	7.01 ± 0.2

Autoclave at 121°C for 20 minutes. Cool to 50°C and add 1.0 ml of polymyxin-B sulphate solution containing 1,00,000 units.

Decontaminate the surface of egg with alcohol/spirit and flame it. Break it asceptically, take out yolk and mix with an equal volume of isotonic (0.85 % w/v) saline. Filter the mixture through sterile gauge, distribute into sterile bottles and heat at 60°C for 30 minutes. Add 100 ml per 900 ml of above basal medium asceptically.

4. <u>MR test medium</u> :

Peptone	5.0 g
K_2HPO_4	5.0 g
Distilled water	1000 ml
Steam until the solids are dissolved, filter and adjust to pH	7.5
Glucose	5.0 g

Add the glucose, mix and distribute 1.5 ml volumes into tubes. Sterilize at 115°C for 10 minutes.

5. Nitrate broth :

Potassium nitrate	1.0 g
Nutrient broth	1000 ml

Autoclave at 115°C for 20 minutes.

6. Nutrient broth :

Peptone	10.0 g
Beef extract	10.0 g
NaCl	5.0 g
Distilled water	1000 ml
pH	7.2 - 7.4

Autoclave at 115°C for 20 minutes.

7. Physiological saline :

8.5 gm of sodium chloride in 1 litre of distilled water. Autoclaved at 15 lbs presure for 20 minutes.

8. Semi-solid agar (SSA) :

Peptone	10.0 g
Beef extract	10.0 g
NaCl	5.0 g
Agar	6.0 g
Distilled water	1000 ml

Dissolve the ingredients other than agar by heating in the water. Adjust pH to 8.0 to 8.4 with 10N NaOH and boil for 10 minutes. Filter, adjust pH to 7.2 to 7.4. Add agar and sterilize at 115°C for 20 minutes.

9. Simmon's citrate :

NaCl	5.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
Citric acid	2.0 g
Distilled water	1000 ml
pH	6.8
Bromothymol blue (0.2 % solution)	40.0 ml
Agar	20.0 g

Autoclave at 121°C for 20 minutes

10. Starch agar :

Potato starch	10.0 g
Distilled water	50 ml
Nutrient agar	1000 ml

Triturate the starch with water to a smooth cream and add to the molten nutrient agar. Mix and sterilize at 115°C for 10 minutes. Distribute into petridishes.

Notes: This medium should not be filtered after adding the starch suspension.

Over heating may hydrolyse the starch.

11. VP test media :

Glucose peptone medium

Peptone	10.0 g
Glucose	5.0 g
Distilled water	1000 ml

Mix and dissolve by gentle heating, filter, adjust to pH 7.6 and distribute into tubes, sterilize at 115°C for 10 minutes in a solid-bottomed container.

12. Acid mercuric chloride :

Mercuric chloride	12.0 g
Distilled water	80 ml
Conc. HCl	16 ml

Mix mercuric chloride with water, add acid and shake well until solution is complete.

13. Bromothymol blue indicator :

Bromothymol blue	2.0 g
N/10 Sodium hydroxide	25.0 ml
Distilled water	475.0 ml

14. Kovac's reagent :

Paradimethylaminobenzal dehyde	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (conc.)	25.0 ml

15. Lugol's iodine :

Iodine	5.0 g
Pot. iodide	10.0 g
Distilled water	100 ml

Dissolve the KI and iodine in 10 ml of water and adjust to volume with distilled water.

For use dilute 1/5 with distilled water.

16. Nitrate test reagents :

Solution A :

Sulphanilic acid	8.0 g
5N acetic acid	1000 ml

Solution B :

Alpha-naphthol	5.0 g
5N acetic acid	1000 ml

17. Phenol reagent (BDH) :

Five parts of commercially available reagent is mixed with 4.7 parts of distilled water to obtain a solution of required acidity.

Appendix - I

Agglutination reaction between the H-antigen of such isolates of *B. cereus* which gave multiple reactions/ no reaction with antisera (Chawmin)

Antigen No.	Agglutination titre with antisera																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
62	160	20	-	40	80	40	80	640	160	160	320	80	320	160	-	-	160	40	40	40
65	80	80	-	40	40	80	20	80	80	40	40	80	320	20	160	40	160	80	20	320
74	-	40	-	20	20	320	80	20	20	40	80	20	40	40	80	320	20	640	160	320
79	-	20	-	-	20	-	-	40	40	80	20	-	40	1280	-	2560	640	320	320	2560
113	160	2560	2560	640	640	160	160	640	640	80	-	40	20	40	320	1280	40	20	20	40
132	20	20	160	160	20	80	40	80	80	80	320	320	40	160	1280	1280	20	320	20	160
136	160	160	40	80	160	160	80	160	5120	640	640	2560	5120	160	1280	2560	160	320	320	640
138	80	80	-	80	80	-	40	640	80	80	160	160	80	160	-	-	80	20	80	20
140	-	-	-	-	-	-	20	20	160	40	320	640	80	20	40	20	80	40	640	40
142	80	40	80	160	320	160	80	40	640	320	80	320	-	80	-	40	320	160	320	160
152	80	40	160	40	80	40	40	40	40	80	40	160	80	80	40	160	40	40	40	80
160	160	40	20	40	40	80	80	80	160	320	80	320	320	160	20	40	80	160	160	160
167	-	40	40	20	20	40	40	20	20	20	20	20	40	20	40	-	20	20	20	20

Appendix - II

Agglutination reaction between the H-antigen of such isolates of *B. cereus* which gave multiple reactions/ no reaction with antisera (fried rice).

Antigen No.	Agglutination titre with antisera																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
83	-	-	20	-	-	-	-	-	40	20	-	-	20	-	160	320	-	-	20	20
100	2560	2560	1280	1280	1280	80	5120	1280	2560	1280	1280	-	1280	1280	-	-	-	5120	1280	2560
103	-	80	160	160	-	20	-	40	-	-	-	40	-	-	160	-	40	20	40	40
104	-	40	640	160	-	-	-	-	-	-	-	-	-	20	160	-	-	-	-	-
105	-	2560	-	2560	-	-	5120	-	-	5120	-	2560	2560	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2560	-	-	-	2560	-
123	-	-	-	-	1280	1280	-	-	-	-	-	-	-	-	-	-	-	-	-	-
137	20	40	-	20	20	40	40	160	30	160	20	640	320	20	160	160	20	40	20	40
166	160	40	40	-	[illegible]	[illegible]	40	80	[illegible]	[illegible]	[illegible]	80	160	80	160	-	320	160	160	640

Appendix - III

Agglutination reaction between the H-antigen of such isolates of *B. cereus* which gave multiple reactions/ no reaction with antisera (skimmed milk powder).

Antigen No.	Agglutination titre with antisera																			
	1	2	5	6	7	8	11	13	14	15	17	18	21	24	26	75	Respa	FM	HSP_2	RE_2
7	-	-	-	-	-	-	-	20	-	20	20	-	-	-	80	640	40	-	-	-
38	80	160	320	640	40	640	80	640	160	320	160	40	160	160	80	640	40	20	-	40
53	160	40	2560	80	80	80	40	320	40	640	1280	80	640	640	320	-	2560	640	640	640
80	320	-	-	20	40	640	1280	1280	640	2560	640	1280	640	2560	-	1280	2560	40	20	80
89	1280	160	-	20	20	5120	2560	1280	320	2560	5120	640	20	5120	640	1280	1280	1280	20	80