List of Publications

Roy, A., Moktan, B. and Sarkar, P.K. (2004) Microbiological quality of dosa marketed in West Bengal, India. Proceedings of the National Seminar on '*Emerging Trends in Applied Botany, Seed Science and Technology*, University of Mysore, Mysore, Nov. 4-6, 2004

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Roy, A., Moktan, B. and Sarkar, P.K. (2007) Microbiological quality of legume-based traditional fermented foods in West Bengal, India. *Food Control* **18**, 1405-1411

Roy, A., Moktan, B. and Sarkar, P.K. (2007) Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods. *Food Control* **18**, 1555-1564

Roy, A., Moktan, B. and Sarkar, P.K. Survival and growth of foodborne bacterial pathogens in fermenting dough of wadi, a legume-based traditional food. *International Journal of Food Science and Technology* (*Submitted for publication: Manuscript No. IJFST-2006-01947*)

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Roy, A., Moktan, B. and Sarkar, P.K. Survival and growth of foodborne bacterial pathogens in fermenting batter of dhokla. *Journal of Food Science and Technology* (*Submitted for publication: Manuscript No. 24/07*)

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Microbiological quality of legume-based traditional fermented foods marketed in West Bengal, India

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Abstract

A total of 105 samples of six different types of legume-based popular fermented foods, namely amriti, dhokla, dosa, idli, papad and wadi, purchased from retail outlets in West Bengal, was analysed to determine their microbiological safety status. While dhokla and idli were of high-moisture foods (62 g (100 g)$^{-1}$), others had a lower moisture level (14–27 g (100 g)$^{-1}$). Papad was alkaline (pH 8.7), whereas all the other foods were acidic (pH 4.4–5.8). Every sample was found contaminated with total aerobic mesophilic bacteria (detection limit, 10 cfu g^{-1}); 38% (40/105) of the samples contained more than 10^6 cfu g^{-1}. Aerobic mesophilic bacterial spores were found in 88% (92/105) of the samples (detection limit, 100 cfu g^{-1}), whereas their anaerobic counterparts were present in 39% (41/105) of the samples (detection limit, 10 cfu g^{-1}). Although all the samples, excepting one, were free from *Staphylococcus aureus* (detection limit, 100 cfu g^{-1}), 20% (21/105) of the samples were found contaminated with *Bacillus cereus* (detection limit, 100 cfu g^{-1}). Enterobacteriaceae were found in 46% (48/105) of the samples (detection limit, 10 cfu g^{-1}). Of the Enterobacteriaceae isolates, 92% were coliforms and 57% were faecal coliforms. *Escherichia coli* (detection limit, 10 cfu g^{-1}) was found in only one sample each of wadi and idli, at a load of 10^3–10^4 g^{-1}. *Salmonella* (detection limit, 1 cell (25 g)$^{-1}$) occurred in 12 samples of wadi, idli and papad, however was absent in the other three products. *Clostridium perfringens* (detection limit, 10 cfu g^{-1}) and *Shigella* (detection limit, 1 cell (25 g)$^{-1}$) could not be detected. The results obtained in the present study indicated that these foods were manufactured using poor-quality starting materials, processed under unhygienic conditions, or/and temperature-abused during transportation and storage. Based on these results, a guideline is recommended for obtaining safe products.
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Keywords: Legume-fermented food; Foodborne pathogens; Microbiological safety

1. Introduction

Fermentation, an old and economical method of producing and preserving food, is widely practised in Asia and Africa (Campbell-Platt, 1987; Steinkraus, 1996). Fermented foods have generally been considered as less likely to be vehicles for foodborne infection or intoxication than fresh foods due to the competitive activity and metabolites of the functional microflora (Nout, 1994). But due to unhygienic handling, external contamination, contaminated water and inferior quality of raw material, many of these foods may get contaminated by bacteria such as *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella*, *Shigella* and many other pathogens. In most of the fermented foods, especially in lactic acid bacterially fermented ones, the inhibition of growth of bacterial pathogens is common and can often ensure safety where levels of contamination are low (Adams & Nicolaides, 1997). But with infectious pathogens, particularly those with a small low infectious dose, some degree of inactivation may be necessary to provide an acceptable level of safety (Beumer, 2001).

In India, legumes alone or in combination with cereals, like rice constitute the basic ingredient of many fermented

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Table 1
Legume-based traditional fermented foods marketed in West Bengal

Food	Substrate	Functional microflora[a]	Product marketed as	Nature of the product	Mode of consumption	Reference
Amriti	Legume	No data available	Ready-to-eat	Syrup-filled, ring-shaped confectionery	Snack	Nil
Dhokla	Legume	LAB, yeast	Ready-to-eat	Steamed, spongy cake	Snack	Desai and Salunkhe (1986); Joshi et al. (1989); Aidoo et al. (2006); Nout et al. (in press)
Dosa	Legume-rice mixture	LAB, yeast	Ready-to-eat	Thin, highly seasoned, griddled pancake	Snack	Soni and Sandhu (1999); Aidoo et al. (2006); Nout et al. (in press)
Idli	Legume-rice mixture	LAB, yeast	Ready-to-eat	Steamed, spongy cake	Snack	Soni and Sandhu (1999); Aidoo et al. (2006); Nout et al. (in press)
Papad	Legume	Yeasts	Raw	Thin, circular wafer	Deep-fried in oil or roasted, or made to condiment	Shurpalekar (1986); Aidoo et al. (2006); Nout et al. (in press)
Wadi	Legume	LAB, yeasts	Raw	Hollow, brittle, balls or cones	Briefly fried in oil, and made to condiment	Sandhu and Soni (1989); Aidoo et al. (2006); Nout et al. (in press)

[a] LAB, lactic acid bacteria.

foods. In the State of West Bengal in India, fermented foods like amriti, papad and wadi are popular from time immemorial. In the passage of time, southern and western Indian fermented foods, like idli, dosa and dhokla have also become the choice for restaurant hunters as nutritious and delicious light midday meal (Table 1). The traditional method of preparation of amriti (Fig. 1) and appearance of the product are similar to those of jalebi, excepting that in pretzel-looked jalebi, blackgram dal (dehusked split beans) is replaced by refined wheat flour (*maida*) and the fermentation time is longer (14–16 h) (Campbell-Platt, 1987; Steinkraus, 1996). The preparation of dhokla (Fig. 2), dosa and idli are similar, excepting that in dosa and idli Bengalgram is substituted with blackgram, and while dosa is a highly seasoned griddled pancake, idli is a steamed pancake resembling dhokla. While all the aforementioned ones are RTE (ready-to-eat) foods, marketed papad (Fig. 3) and wadi (Fig. 4) are cooked before consumption.

Microbiological safety of fermented foods is an important issue in developing countries, including India. Processing technologies that ensure food safety are required at

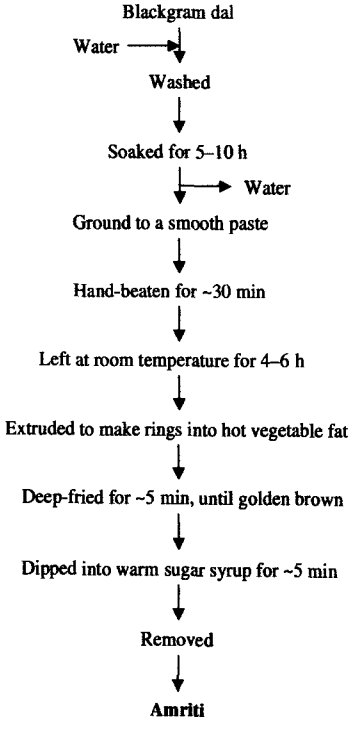

Fig. 1. Flow sheet for amriti production.

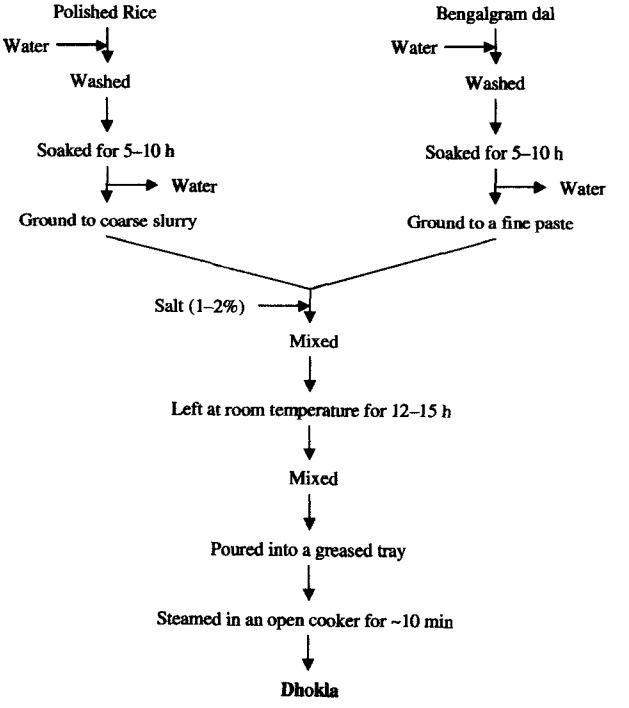

Fig. 2. Flow sheet for dhokla production.

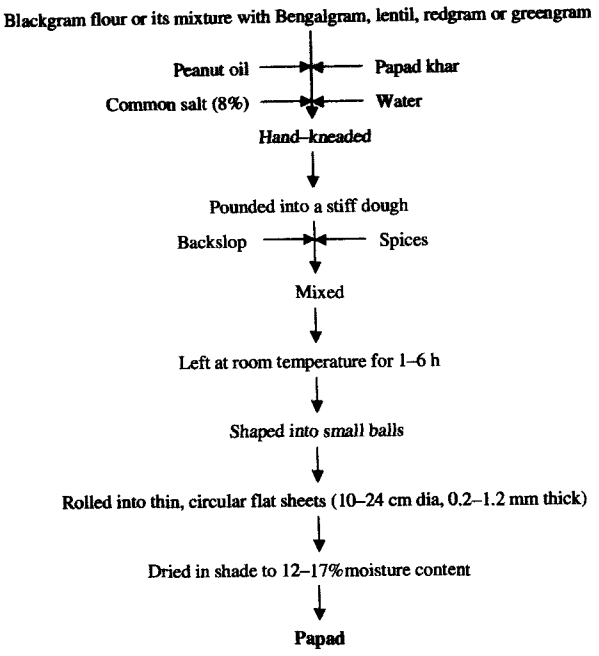

Fig. 3. Flow sheet for papad production.

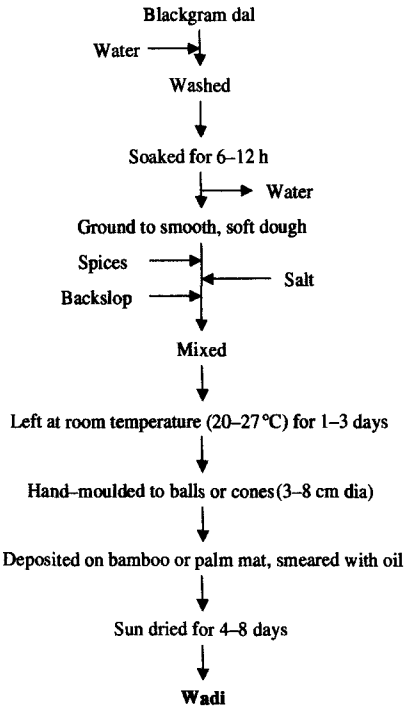

Fig. 4. Flow sheet for wadi production.

both the rural and urban levels, particularly in view of the frequently poor sanitary conditions and high ambient temperatures. The production of safe foods is the responsibility of the producers. But the authorities need to regularly verify and validate those through inspection and product testing both at the site of production and at the point of sale. These foods have not so far been studied with regard to their microbial safety. The present study was hence undertaken to evaluate the microbiological quality of legume-based fermented foods retailed in West Bengal, and to suggest a safety guideline for these products on the basis of the explored status.

2. Materials and methods

2.1. Materials

The culture media, chemicals and sampling bags used were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India, unless mentioned otherwise.

2.2. Reference organisms

B. cereus ATCC9139 was obtained from Dr. M.J.R. Nout, Wageningen University, The Netherlands, and *S. aureus* MTCC96, *C. perfringens* MTCC450, *E. coli* MTCC118, *Salmonella typhi* MTCC733 and *Shigella flexneri* MTCC1457 were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

2.3. Sampling

A total of 105 samples belonging to six different kinds of legume-based traditional fermented products were purchased from randomly selected retail outlets and restaurants scattered over different places of the State (Table 2). The unpackaged samples, each having an average weight of 150 g, were collected in sterile Nasco sampling bags (PW389), and kept in an ice-box. The samples of amriti, dhokla, dosa and idli were found to be sold unpackaged. Dosa was found to be sold ready prepared and, hence, sampled immediately after its preparation. Samples of wadi and papad, sold as their sealed packets made with low-density polyethylene film, were also collected. The samples were transported to the laboratory immediately and analysed as early as possible.

2.4. Moisture content and pH

The moisture content was determined by drying approximately 10 g sample of foods at 105 ± 1 °C in a hot air oven to constant weight using a Sartorius CP224S (Sartorius AG, Göttingen) balance (Banerjee & Sarkar, 2003).

A 10 g sample was blended with 20 ml carbon dioxide-free deionized water, and pH of the slurry was determined using a pH meter type 335 (Systronics, Naroda).

2.5. Microbiological analysis

The methods used were based on those followed by FDA (1984), Speck (1984), Nout, Bakshi, and Sarkar (1998) and Banerjee and Sarkar (2003). Representative samples (10 g) were homogenized with 90 ml sterile peptone-physiological saline (0.1% w v^{-1} neutral peptone, 0.85% w v^{-1}

Table 2
Sampling of legume-based fermented foods from retail shops in different districts of West Bengal

Food	District[a] of collection	No. of samples analysed		
		Total	Unpackaged	Packaged[b]
Amriti	C, D, M_1, N_3	8	8	L-0, B-0
Dhokla	D, N_3	5	5	L-0, B-0
Dosa	C, D, J, K, M_1, N_3	16	16	L-0, B-0
Idli	C, D, J, K, M_1	13	13	L-0, B-0
Papad	C, D, H_1, H_2, J, K, M_1, M_2, N_1, N_2, N_3, S, W	29	1	L-1, B-27
Wadi	B_1, B_2, C, D, E, H_1, H_2, J, K, M_1, M_2, N_1, N_2, N_3, S, W	34	23	L-11, B-0

[a] B_1, Bankura; B_2, Bardhaman; C, Cooch Behar; D, Darjiling; H_1, Hooghly; H_2, Howrah; J, Jalpaiguri; K, Kolkata; M_1, Malda; M_2, Murshidabad; N_1, North 24 Parganas; N_2, Nadia; N_3, North Dinajpur; S, South 24 Parganas; W, West Midnapore.
[b] L, locally packaged; B, branded.

sodium chloride, pH 7.2) using a Stomacher lab-blender 400 (Seward Medical, London) at 'normal' speed (2 min for wadi, 1 min for others). Duplicate counting plates were prepared using appropriate dilutions. For pour-plating, 1 ml of the dilution was mixed with molten (45 °C) medium. For spread-plating, 0.1 ml of the dilution was spread on the surface of a dried plate. After incubation, the colonies appearing on the selected plates were counted and calculated as colony forming units (cfu) per gram fresh weight sample. The representative colonies of each type were picked up and diluted by streaking out. After microscopic examination, the purified colonies were grown on slants or in broths of suitable media and stored at 4 °C.

Total aerobic mesophilic bacteria were enumerated by pour-plating using plate count agar (M091) and incubating at 35 °C for 18–24 h. For the estimation of mesophilic bacterial spores, 10% (w v^{-1}) sample suspension was heated at 80 °C for 30 min, suitably diluted, and spread on PCA plates followed by incubation at 30 °C for 72 h (for aerobic sporeformers) and pour-plated with perfringens agar (M579, FD011 and FD012) followed by incubation at 37 °C for 48 h in an anaerobic jar with AnaeroHiGas pack (LE002A) (for anaerobic sporeformers) (Banerjee & Sarkar, 2003).

Enumeration of *B. cereus* was made on spread-plates of *B. cereus* selective agar (M833, FD003 and FD045), incubated at 35 °C for 24–48 h. A representative number of presumptive isolates was confirmed on the basis of motility, endospore formation, glucose fermentation, acetylmethylcarbinol production and nitrate reduction.

Isolation of *S. aureus* was carried out on spread-plates of Baird-Parker agar (M043, FD047 and FD045), incubated at 35 °C for 24–48 h. Representative isolates were confirmed by the production of coagulase and acid from mannitol using coagulase mannitol broth base (M277) with appropriate addition of sterile pre-tested coagulase plasma, thermostable DNase using DNase test agar with toluidine blue (MI041) and production of acetylmethylcarbinol.

Selective enumeration of *C. perfringens* was done in pour-plates of perfringens agar, incubated at 37 °C in an anaerobic jar for 18–48 h. The representative isolates, maintained in cooked meat medium (M149), were confirmed by testing motility and nitrate reduction using motility nitrate medium (M630I), raffinose fermentation using raffinose gelatin medium (M987, substituting lactose with raffinose), and lactose fermentation and gelatin liquefaction using modified lactose gelatin medium (M987).

Enterobacteriaceae members were isolated by mixing appropriate dilutions of samples with tryptone soya agar (M290) and incubating the plates at room temperature (27 °C) for 1–2 h followed by a thick overlay of violet red bile glucose agar without lactose (M581) and incubated at 35 °C for 18–24 h. The presumptive isolates were confirmed on the basis of cytochrome oxidase using oxidase disc (DD018) and glucose fermentation in stab cultures of purple agar base (M098) supplemented with 1% w w^{-1} D (+) glucose (Merck 17809).

For the detection of coliforms, brilliant green bile broth, 2% (M121) with inverted Durham tubes was inoculated with confirmed Enterobacteriaceae isolates, incubated at 37 °C for 24–48 h, and examined for gas formation. For tests of faecal coliforms, inoculated broth tubes were incubated at 44 °C for 24 h. The presence of *E. coli* was confirmed on the basis of indole production by using tryptone water (M463I) and Kovac's reagent strip (DD019).

For qualitative detection of *Salmonella* and *Shigella*, 25-g samples were added to 225 ml buffered peptone water (M614), followed by incubation at 35 °C for 20 h. Ten and 0.1 ml of this pre-enrichment culture were added to fluid selenite cystine medium (M025A) and Rappaport–Vassiliadis medium (M880), respectively. The former was incubated at 35 °C for 24 h, whereas the latter at 42 °C for 18–48 h. Bismuth sulphite agar (M027) plates, streaked with the enriched broth cultures, were incubated at 35 °C for 24–48 h. The presumptive isolates were confirmed on the basis of acid and gas production by using triple sugar iron agar (M021), lysine iron agar (M377), motility by using motility nitrate medium (M630I), production of acid from glucose by using MRVP medium (M070), and production of indole by using tryptone water and Kovac's reagent strip.

2.6. Statistical analysis

Statistical treatment of the data was performed using SPSS 12.0 for Windows for standard error of measurements (SEM) and analysis of variance (ANOVA).

3. Results and discussion

The results of moisture, pH and microbial analyses of 105 samples of six different kinds of legume-based traditional fermented foods marketed in West Bengal are summarized in Table 3. While dhokla and idli were the high-moisture (62 g $(100\,g)^{-1}$) foods, others contained less moisture (14–27 g $(100\,g)^{-1}$). Papad was alkaline, whereas all the other foods were acidic.

Thirty-eight percent (40/105) of the samples contained total aerobic mesophilic bacterial cells at a level of $>10^6$ cfu g^{-1}. Majority of the samples of each of the six foods, except dosa, had a high count ($>10^4$ cfu g^{-1}) of these bacteria, indicating a lapse in good hygiene practices followed in preparing these foods. While most of the samples of amriti, dosa, idli and papad contained total aerobic mesophilic bacteria in the range of 10^2–10^6 cfu g^{-1}, in most of the samples of dhokla and wadi their count was at a higher level ($>10^6$ cfu g^{-1}). Since marketed amriti, dosa and idli are either fried or steamed at the final stage of their preparation, death of most of the fermenting organisms and associated microflora (excepting those occurring in 'cool pockets') and a consequent low count of total aerobic mesophilic bacteria were expected in those samples. Although marketed dhokla is a steamed product, their high count ($>10^6$ cfu g^{-1}) in 100% of the samples might be mostly due to post-preparation contamination introduced from seasoning ingredients, including spices, chilly, grated coconut and curry (*Murraya koenigii*) leaves accompanied with a high moisture content of the product. Plate count agar is a non-selective complex medium commonly used for enumerating the total microbial content in foods. So, the viable count in the samples of papad and wadi, which did not pass through any heat treatment process, was likely of fermenting microorganisms along with associated contaminating microflora.

The external surface of the cereal grains is heavily contaminated with saprophytes acquired during development of the plants along with contaminants from soil, air, animals and also humans (Sarrias, Valero, & Salmerón, 2002). *Bacillus* spp. are important as food-spoilage organisms, and can be isolated from a variety of animal and plant products (Johnson, 1984). Aerobic mesophilic bacterial spores were found in 88% (92/105) of the samples. All the samples of amriti, dhokla and papad contained these spores. A high count (10^5 cfu g^{-1}) of them was found in papad and wadi (10/63 samples). As dosa samples were freshly prepared ones, their load in the product was never more than 10^5 cfu g^{-1}. On the other hand, the load of their anaerobic counterpart was less; they occurred in 39% of the tested samples (41/105). Amriti, dosa and idli were free of them.

Table 3
Moisture content, pH and levels of microflora (expressed as percentages of samples analysed) of legume-based traditional fermented foods marketed in West Bengal

Parameter	Amriti ($n=8$)	Dhokla ($n=5$)	Dosa ($n=16$)	Idli ($n=13$)	Papad ($n=29$)	Wadi ($n=34$)
Moisture, g $(100\,g)^{-1}$[a]	19.5bc ± 1.12	62.1a ± 0.86	27.3b ± 1.73	61.8a ± 1.58	18.0bc ± 0.41	14.4c ± 0.33
pH[a]	5.8b ± 0.07	4.9c ± 0.11	4.4d ± 0.08	4.6cd ± 0.07	8.7a ± 0.07	5.7b ± 0.04
Bacterial load (cfu g^{-1} fresh weight)						
Total aerobic mesophilic bacteria						
<Detection limit (10)						
10^2–10^4	12.5		56.3	23.1	17.2	2.9
$>10^4$–10^6	62.5		43.7	76.9	75.9	5.9
$>10^6$–10^9	25	60			6.9	55.9
$>10^9$–10^{12}		40				35.3
Aerobic mesophilic bacterial spores						
<Detection limit (100)			50	15.4		8.8
10^2–10^5	100	100	50	84.6	79.3	76.5
$>10^5$–10^7					20.7	14.7
Anaerobic mesophilic bacterial spores						
<Detection limit (10)	100	40	100	100	44.8	35.3
10–10^4		60			55.2	47.1
$>10^4$–10^5						17.6
Bacillus cereus						
<Detection limit (100)	75	40	81.3	92.3	79.4	82.4
10^2–10^3		20	12.4	7.7	10.3	8.8
$>10^3$–10^4	25		6.3		6.9	2.9
$>10^4$–10^6		40			3.4	5.9
Enterobacteriaceae						
<Detection limit (10)	50	80	87.5	46.2	69	26.5
10–10^3	25			7.6	17.2	17.6
$>10^3$–10^5	12.5		12.5	46.2	13.8	23.5
$>10^5$–10^8	12.5	20				32.4

[a] Values are mean with standard error of measurements. Means with the same following letters, within rows, are not significantly different ($P<0.05$).

Table 4
Percentage of Enterobacteriaceae isolates containing coliform, faecal coliform and *E. coli*.

Enterobacteriaceae component	Food						
	Amriti	Dhokla	Dosa	Idli	Papad	Wadi	Total
Coliform	49.1	100	58.7	82.6	49.2	59.8	91.9
Faecal coliform	0	66.7	0	15.5	12.5	18.6	56.9
E. coli	0	0	0	3.9	0	0.6	0.1

All the six types of foods contained *B. cereus*; this organism occurred in 20% of the samples (21/105). The potentially hazardous level ($>10^4$ cfu g^{-1}) was observed in dhokla, papad and wadi. The presence of considerable levels of *B. cereus* was recorded in several legume-fermented foods, such as Indonesian tempe (Samson, Van Kooji, & De Boer, 1987), African dawadawa (Antai & Ibrahim, 1986) and Indian kinema (Nout et al., 1998). The presence of this organism at high levels suggests a potential risk of these foods to the consumer, because of the subsequent production of toxin associated with food poisoning (Banerjee & Sarkar, 2004). However, it was found in a legume food (kinema) that in presence of fermenting microorganisms (*B. subtilis*), the growth of *B. cereus* was adversely affected and the production of enterotoxin was ceased (Nout et al., 1998). *C. perfringens* could not be detected from any of the 105 samples, and only one sample was found contaminated (at the load of 4×10^4 cfu g^{-1}dhokla) with *S. aureus*. The latter organism was also not detected in kinema (Nout et al., 1998). Possibly, the lack of initial contamination, or the impact of competition and/or antagonistic reactions would have prevented its proliferation. It may also happen that legumes do not offer a suitable environment for the proliferation of *S. aureus*.

Enterobacteriaceae counts are considered more generally as an indicator of hygienic quality rather than of faecal contamination, and therefore say more about general microbiological quality than possible health risks posed by the product (Adams & Moss, 1995). Enterobacteriaceae occurred in all the six types of foods studied (Table 4); these were detected in 46% (48/105) of the samples. Of the Enterobacteriaceae isolates, 92% were coliforms and 57% were faecal coliforms. The occurrence of these microorganisms in a food is considered as a reflection of the process practised during its preparation and/or subsequent handling under inefficient hygienic condition (ICMSF, 1978). The presence of a high count of faecal coliforms in dhokla, papad, wadi and even freshly prepared idli indicates a high risk that other pathogenic organisms have also contaminated the food. One sample each of idli (3.8×10^3 cfu g^{-1}) and wadi (3.2×10^4 cfu g^{-1}) were found contaminated with *E. coli*.

Although the presence of *Salmonella* in foods of animal origin has been well documented, very limited studies are available on vegetarian foods, particularly the processed ones (Yadav, Zende, & Sharma, 2000). More than 95% of cases of infections caused by *Salmonella* are foodborne and these infections account for about 30% of deaths resulting from foodborne illnesses (Hohmann, 2001). *Salmonella* was present in 11.4% of the total samples analysed (12/105). It was not detected in amriti, dhokla and dosa. However, its prevalence in the other three foods is noteworthy; 15% (2/13), 14% (4/29) and 18% (6/34) of the samples of idli, papad and wadi, respectively, were found contaminated with this pathogen. Since marketed idli is RTE, presence of *Salmonella* in idli is alarming. However, it will be killed during heat-processing for consumption of papad and wadi. *Shigella* could not be detected in any of the samples.

The microbial composition of these products indicates that their manufacturing processes did not support survival and growth of *S. aureus*. On the other hand, a high level of total aerobic mesophilic bacterial cells in majority of the samples indicates either that highly contaminated substrates were used or that poor processing practices e.g., inappropriate handling or unhygienic condition were involved, as was observed during the study with sufu (Han, Beumer, Rombouts, & Nout, 2001). Considering that no sign of spoilage was recorded, it might be assumed that most mesophilic bacterial spores either did not germinate or were not metabolically active in these products.

Detection of the presence of a high count of total aerobic mesophilic bacteria, *B. cereus*, and Enterobacteriaceae in all these foods suggests that a better control is needed and that some changes in the manufacturing practices, storage, distribution and service should be made to enhance their microbial safety.

Among the critical control points (CCPs) are raw materials, water, beating or mixing batter or dough with bare hands, utensils, drying environment (in case of papad and wadi), post-preparative storage conditions, and dish cloth. Many times, uncleaned raw materials (rice, blackgram and Bengalgram) are used. In most cases, due to lack of running water availability producers store water under vulnerable conditions subject to contamination. Personal cleanliness is another aspect which can reduce foodborne hazards; use of gloves at the time of beating or mixing batter or dough reduces the chance of cross-contamination. Utensils represent an important source of food contamination, since the same utensils are used in different batches of preparation without any in-between cleaning. Most of the fermented foods under study are taken ready to eat for sale and are, therefore the most susceptible to microbial growth in view of the longer length of time between preparation and consumption under improper temperature conditions (30–38 °C). Open-air drying of papad and wadi leads exposure of these foods to aggravating environmental conditions, such as the presence of insects, rodents, other animals and dust. The

dish cloth used in several tasks represents another hazard to the safety of foods. Hence, training of people, and producers and sellers in particular, for a cultural change would be one of the most effective interventions to reach a safer food.

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Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods

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Abstract

An antibiogram of 48 strains of *Bacillus cereus* isolated from 6 different kinds of legume-based Indian fermented foods (amriti, dhokla, dosa, idli, papad and wadi) was generated against 18 different antibiotics that are commonly used against foodborne diseases, mainly gastroenteritis. Each of the isolates was found to be resistant against at least nine different antibiotics. Production of extracellular enzymes, namely protease, lipase and amylase by 33%, 27% and 46%, respectively, of the isolates indicates their potentiality for food spoilage. In brain–heart infusion broth supplemented with glucose, the $D_{100\,°C}$-values of the tested 12 strains ranged from 3.0 to 9.2 min. In nutrient broth, the minimum and maximum pHs permitting growth of *B. cereus* were 5.3 and 11.6, respectively. The minimum inhibitory concentrations of sodium chloride, benzoic acid and sorbic acid for the growth of the isolates were 65–85 mg ml^{-1}, 400–700 μg ml^{-1} (pH 5.0–4.2) and 500–600 μg ml^{-1} (pH 5.0–4.8), respectively. Of the tested 10 strains, eight were resistant to 300 μg nisin ml^{-1} (pH 5.0). While studying the combined effect of selected hurdles on the growth of an isolate, the judicious combination considered was 20 mg sodium chloride, 300 μg benzoic acid and 25 μg nisin ml^{-1} at pH 5.6. The whole cell protein fingerprinting (WCPF) analysis using SDS–PAGE revealed a high level of diversity among the isolates. At ⩾60% similarity level, the WCPF profiles could be grouped into four major clusters which were divided into 34 subclusters. Most of the subclusters were source-wise homogeneous.
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Keywords: *Bacillus cereus*; Antibiotic susceptibility; Extracellular enzyme; *D*-value; Natural preservative; Combined effect; Whole-cell protein fingerprinting; Diversity

1. Introduction

Bacillus cereus is widely distributed in the natural environment and is easily spread to many types of food, especially those of plant origin. It causes food spoilage and two distinct types of food poisoning: the diarrhoeal type and the emetic type. Whereas the former type is caused by complex enterotoxins produced during vegetative growth in the small intestine, the latter type is produced by growing cells in the food (Granum & Lund, 1997).

In spite of competition and antagonistic activity incurred by the dominant fermenting microflora, *B. cereus* has been reported in some legume-based traditional fermented foods, viz. African dawadawa, Indonesian tempeh, and Indian idli and kinema (Antai & Ibrahim, 1986; Nout, Bakshi, & Sarkar, 1998; Samson, van Kooij, & de Boer, 1987; Varadaraj, Keshava, Devi, Dwarakanath, & Manjrekar, 1992). The present authors isolated *B. cereus* from 20% of the samples of legume-based Indian fermented foods (Roy, Moktan, & Sarkar, 2006). This finding triggered them to study the behavioural patterns of *B. cereus* so that measures can be undertaken to control this dreadful pathogen.

Surveillance of antimicrobial resistance is essential for providing information on the magnitude and trends in resistance and for monitoring the effects of interventions, especially because the prevalence of resistance varies widely between and within countries, and over time (WHO, 2001).

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Strains of foodborne bacterial pathogens that are resistant to a variety of antibiotics have become a major health concern (Kiessling et al., 2002). Sixty years of increasing application of antibiotics have created an ecological imbalance – the enrichment of multiple antibiotic-resistant pathogenic bacteria. Finding out an antibiotic resistance profile of the isolated strains against commonly used antibiotics for treating gastroenteritis was one of the objectives of the present work.

Increasing interest by consumers and producers in food safety and quality gives shelf-life evaluation a new significance. Proteolytic, lipolytic and amylolytic activities of bacteria indicate their potentiality for food spoilage (Braun, Fehlhaber, Klug, & Kopp, 1999). Hence, our second objective was to evaluate the production of these enzymes so that their role in spoilage can be predicted.

When foods containing spores of *B. cereus* are cooked, the spores often survive and may be heat-shocked into germination. If these foods are then left to ambient temperature, germination and growth may take place, leading to achieve a competition-free favourable condition causing spoilage of the food and/ or producing emetic toxins. To understand the hazardous potential of the sporeformers which can survive cooking processes, quantification of thermal inactivation of spores of *B. cereus* isolates from these foods was our third objective.

Nowadays, there is strong interest in the use of natural antimicrobials for preservation of minimally processed foods. The addition of appropriate antimicrobial preservatives is used to reduce the growth of microbial contaminants in foods. Benzoic acid is widely used chiefly on account of its low price, whereas sorbic acid is preferred to others because of its physiological harmlessness and organoleptic neutrality (Lueck, 1980). Since nisin does not persist in the body or the environment, nor it is associated with the bacterial resistance to itself, it has the potentiality for widespread use as a food preservative. Hence, our fourth objective was to find out the minimum inhibitory concentrations (MICs) of sodium chloride, benzoic acid, sorbic acid and nisin individually to prevent this potent pathogen. The microbial stability and safety of most foods are based on a combination of several preservative factors (hurdles), which microorganisms present in the food are unable to overcome. Using an intelligent combination of hurdles it is possible to improve not only the microbial stability and safety but also the sensory and nutritive quality as well as economic aspects of a food (Leistner, 1994). Therefore, an attempt was also made to determine the combined effect of pH, salt, one weak acid preservative and nisin on the growth of an isolate.

Molecular typing methods, including both genotypic and phenotypic analyses, could be employed to find out differences among subspecies or strains of *B. cereus*. Among phenotypic methods whole-cell protein fingerprinting (WCPF) using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) may be employed as a useful tool to study the diversity. Therefore, WCPF is considered here as a discriminatory tool for measuring diversity among the isolates at the subspecies level.

2. Materials and methods

2.1. Organisms

The 48 strains of *B. cereus* used were isolated from 105 random samples of 6 different legume-based fermented foods (Table 1), collected from retail outlets in India (Roy et al., 2006). The organisms were maintained on nutrient agar (HiMedia M561) slants at 4 °C with subculturing after every 6 months.

2.2. Susceptibility to antibiotics

An antibiogram was developed using the disc agar diffusion method. Three colonies, grown on tryptone soya agar (HiMedia M290) at 37 °C for 24 h, were transferred to about 5 ml tryptone soya broth (HiMedia M011) and incubated at the same temperature for 6–8 h until the broth became moderately turbid. A sterile cotton swab (HiMedia PW005) was dipped into the inoculum and applied evenly onto Mueller–Hinton agar (HiMedia M173) plate (4 mm thick). After drying for 15 min, various antibiotic susceptibility test discs (HiMedia) were applied aseptically keeping a distance of at least 3 cm between their centres. The plates were incubated at 37 °C for 14–19 h. The zones showing complete inhibition were measured.

Table 1
Food sources of the *B. cereus* strains used

Source	Nature of the marketed product	Isolate no.
Amriti	Deep-fried (~5 min) and syrup-filled (by dipping into warm sugar syrup for ~5 min) ring-shaped confectionery	104-B1, 104-B2, 104-B3, 105-B1, 105-B2, 105-B3
Dhokla	Steamed (in an open cooker for ~10 min), spongy cake	34-B1, 35-B1, 37-B1
Dosa	Seasoned, griddled (for ~5 min) pancake	16-B1, 55-B1, 98-B1, 98-B2, 98-B3
Idli	Steamed (in an open cooker for ~10 min), spongy cake	94-B1, 94-B2, 94-B3
Papad	Shade dried (to 12–17% moisture content), thin, circular wafer	18-B2, 18-B3, 18-B5, 52-B1, 52-B2, 57-B2, 57-B3, 57-B4, 57-B5, 70-B1, 70-B2, 93-B1, 93-B2, 93-B3, 113-B1, 113-B2, 113-B3
Wadi	Sun-dried (for 4–8 days), hollow, brittle cones	2-B1, 2-B3, 6-B2, 46-B2, 49-B1, 49-B2, 66-B1, 66-B2, 66-B3, 66-B4, 66-B5, 111-B1, 111-B2, 111-B3

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2.3. Production of extracellular enzymes

Each of the *B. cereus* isolates was grown in 20 ml brain heart infusion broth (HiMedia M210) at 37 °C for 20 h, and centrifuged (model R-24; Remi Instruments, Mumbai, India) at 9500*g* for 30 min. The supernatant was filtered (0.2 μm cellulose acetate; Sartorius) and stored in a pre-sterilized screw-capped glass tube at 4 °C. A 50 μl aliquot of it was used for determining the activities of different extracellular enzymes using well-assay plate method in suitable media. Production of protease, lipase and amylase was determined using milk agar (HiMedia M163), tributyrin agar base (HiMedia M157) added with 1.0% $v v^{-1}$ tributyrin (Fluka 91,012), and starch agar (HiMedia M107), respectively. The incubated starch agar plates were flooded with Lugol's iodine solution. The results were expressed as clear zone diameter (including well diameter of 5 mm).

2.4. Thermal inactivation of sporeformers

The method followed was based on the one described by Johnson, Nelson, and Busta (1982). Overnight growth of *B. cereus* in tryptone soya broth at 30 °C on a rotary shaker (200 rpm) was spread on dried plates of nutrient agar fortified with different minerals and salts (fortified nutrient agar; Kim & Goepfert, 1971) which were kept upright for 24 h and inverted for an additional 24 h at 30 °C. The plates were then held at 4 °C for 24 h. The growth on each plate was suspended in 10 ml cold sterile distilled water by scraping the surface with a bent glass rod. The suspensions were centrifuged eight times at 9500*g* for 8 min each. Between each centrifugation, the pellets were resuspended in 60 ml cold sterile distilled water. Final pellets were suspended with an appropriate amount of water. The working spore suspensions were subjected to heat-shock at 80 °C for 30 min, and then cooled to 50 °C.

Tubes of 9 ml brain heart infusion broth with 10 g glucose l^{-1} (BHIG) were placed in a water-bath. When the temperature of the monitored uninoculated broth reached 100 °C, 1 ml of the *B. cereus* spore suspension, held at 50 °C (in order to minimize cooling effect), was added to each tube of the test broth and allowed to wait for 35 s in order to return to 100 °C. The tubes in batches were removed from the bath after the specified time interval. Following cooling at room temperature, the tubes were diluted serially at successive decimal levels using ice-cold sterile distilled water. Appropriate dilutions (0.1 ml) were used for plate count on nutrient agar following incubation at 35 °C for 24 h. The *D*-value was computed from the negative reciprocal of the slope of individual trials using the linear portion of the curve of log cfu against time, plotted on a semi-log scale.

2.5. Influence of pH on growth

The pH of sterile nutrient broth (HiMedia M002) was adjusted to different levels using 2 N NaOH/ HCl and a pH meter (model 335; Systronics, Ahmedabad, India). The broth (10 ml) in an Erlenmeyer flask was inoculated with 0.1 ml of a 24 h-old culture. The flasks were incubated on a shaker (200 rpm) at 35 °C for 24 h. The growths were measured turbidimetrically at 580 nm using a spectrophotometer (Systronics type 103).

2.6. Influence of food preservatives on growth

For the determination of MIC, plates of nutrient agar supplemented with different concentrations of sodium chloride, or filter-sterilized (0.2 μm cellulose acetate) benzoic acid (1.00134.0005, E. Merck (India) Ltd., Mumbai, India) (stock solution, 3 mg ml^{-1}) or sorbic acid (1,947,109; Sisco Research Laboratories, Mumbai, India) (stock solution, 2 mg ml^{-1}) were spotted (5 spots per plate) with 18 h-old cultures using a 2 mm-diameter loop (Banerjee & Sarkar, 2004).

A sterile stock solution containing 1 mg nisin ml^{-1} was prepared by dissolving 0.4 g Nisaplin (Aplin & Barret Ltd., Beaminster, Dorset, England) in 10 ml of 0.02 N HCl (pH 1.85), and the pH was adjusted to 3.0 followed by autoclaving at 0.7 kg cm^{-2} for 20 min and filtration through a sterilized Whatman No. 1 paper (Davies et al., 1998). Molten (45 °C) nutrient agar was mixed with appropriate volume of nisin stock solution to get desired concentrations and poured into plates. Fresh cultures (18 h-old) were spotted on the plates (5 spots per plate).

To study combined effects, nutrient broth supplemented with different concentrations of sodium chloride was sterilized by autoclaving. Filter-sterilized benzoic acid (stock solution of 3 mg ml^{-1}) and autoclaved (0.7 kg cm^{-2}, 20 min) and aseptically filtered (Whatman No. 1 paper) nisin (stock solution of 1 mg ml^{-1}) were added separately to sterile nutrient broth to get desired concentrations of those. Each broth was inoculated with 0.1 ml of 18 h-old culture of *B. cereus* 37-B1 in nutrient broth and incubated on a shaker (200 rpm) at 30 °C for 24 h. The growth was measured turbidimetrically at 580 nm.

Next, four variables (pH, sodium chloride, benzoic acid and nisin) with three levels of each (selected from the linear slopes of growth against individual hurdle), 19 different combination sets were prepared as per Hoke's response surface design (Thompson, 1982). Sodium chloride was added to nutrient broth to get appropriate concentrations. After autoclaving, sterile benzoic acid and nisin solutions were added to the sterile sodium chloride-supplemented nutrient broth, and the final pH was adjusted with sterile 2 N NaOH/ HCl. Inoculation and incubation of different sets and monitoring of growth were the same as described above.

2.7. Whole-cell protein fingerprinting

SDS–PAGE was carried out following the method described by Laemmli (1970) in a vertical gel electrophoresis system (Cat No. 05-03, Bangalore Genei, Bangalore,

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India). A solution of 30% (w v^{-1}) acrylamide (SRL 014,022) and 0.8% (w v^{-1}) *N*,*N*-methylene bisacrylamide (SRL 134,985) (solution A) was prepared. While 3.0 M Tris (hydroxymethyl)aminomethane (Tris-HCl; SRL RM262), pH 8.9 was used as resolving gel buffer, 0.5 M Tris-HCl, pH 6.8 served as stacking gel buffer. A 10% (w v^{-1}) solution of running gel was prepared by taking appropriate volume of solution A, resolving gel buffer, 10% (w v^{-1}) sodium lauryl/dodecyl sulphate (SDS; SRL 1948101) and distilled water. Excess *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED; SRL 202788) and a pinch of ammonium persulphate (APS; SRL 0148134) were added to set the gel suitably. When the resolving gel set in, 4% (w v^{-1}) stacking gel, prepared by taking appropriate volume of solution A, stacking gel buffer, 10% SDS, distilled water, TEMED and APS, was poured over the resolving gel.

Samples were prepared by taking a loopful of culture from a 24 h-old (37 °C) nutrient agar plate and washing it three times in phosphate buffer-saline (0.2 M, pH 7.0) by centrifugation at 3000*g* for 15 min each. The pellet was suspended in stacking gel buffer and boiled in a boiling water-bath for 10 min after adding equal volume of 2x sample buffer containing 20% (v v^{-1}) β-mercaptoethanol (SRL 1327198). A discontinuous buffer system was used. The tank buffer was made up of 0.025 M Tris, 0.192 M glycine (Merck India 4201), 0.1% SDS (pH 8.3). Samples were stacked at constant current of 15 mA and resolved at 25 mA until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was fixed in 10% v v^{-1} glacial acetic acid (Merck 60006325001046) for 30 min, stained with coomasie brilliant blue R250 (SRL 024018) solution for 12 h and washed in a destaining solution (methanol (SRL 132977) : acetic acid : water :: 4:1:5) until the protein bands became clearly visible in a colourless gel matrix. The gels were photographed and then processed using the NTSYS pc. 2.0 software for generation of the cluster analysis in a dendrogram based on Dice's similarity coefficient (S_D) and the unweighted pair group method using arithmetic averages (UPGMA).

2.8. Statistical analysis

Experimental data were analysed statistically using Microsoft Excel and SPSS v. 12.0.

3. Results

3.1. Susceptibility to antibiotics

The results for susceptibility of the 48 strains *B. cereus* to 18 different antibiotics, including β-lactams (5), benzene derivative (1), aminoglycosides (2), macrolides (2), peptides (2), glycopeptide (1), quinolones (2), nitro-imidazole (1), tetracycline and trimethoprim, are shown in Table 2. Peptide, glycopeptide, trimethoprim, metronidazole and most of the β-lactam antibiotics had no inhibitory action on the growth of the isolates. Most of the isolates were resistant to antibiotics inhibiting prokaryotic cell wall synthesis, however were sensitive to those inhibiting protein synthesis.

3.2. Production of extracellular enzymes

The results on the production of three extracellular enzymes, viz. protease, lipase and amylase are presented in Table 3. Proteolytic and amylolytic activities were found in 33% and 46%, respectively, of the isolates. However, lipolytic activity was found in only 27% of the isolates. Eleven

Table 2
Antibiogram of *B. cereus* strains ($n = 48$) isolated from cereal based Indian fermented foods

Mechanism of action	Antibiotics (disc^{-1})	Percent score[a]		
		Sensitive	Intermediate	Resistant
Inhibition of cell wall synthesis	Ampicillin (10 μg)			100
	Bacitracin (10 U)		29	71
	Carbenicillin (100 μg)			100
	Cephalothin (30 μg)	2		98
	Cloxacillin (10 μg)			100
	Penicillin G (10 U)			100
	Vancomycin (10 μg)			100
Inhibition of protein synthesis	Chloramphenicol (30 μg)	96	4	
	Erythromycin (15 μg)		60	40
	Kanamycin (30 μg)	58	40	2
	Streptomycin (10 μg)	67	29	4
	Tetracycline (30 μg)	83	17	
Damage to cell membrane	Polymyxin B (300 U)			100
Inhibition of nucleic acid synthesis	Ciprofloxacin (10 μg)	98		2
	Nalidixic acid (30 μg)	38	58	4
	Rifampicin (15 μg)	6	25	69
	Metronidazole (5 μg)			100
Inhibition of folic acid synthesis	Trimethoprim (10 μg)			100

[a] The inhibition zone size (diameter in mm) interpretation was according to Banerjee and Sarkar (2004).

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Table 3
Production of extracellular enzymes by *B. cereus* isolates ($n = 48$) from different food sources

Source	Isolate no.[a]	Zone diameter (mm)[b]		
		Protease	Lipase	Amylase
Amriti	104-B3	27		30
	105-B2	26		11
	105-B3	32		33
Dhokla	34-B1	42	25	31
	37-B1			24
Dosa	55-B1		32	
Idli	94-B1			22
	94-B2			14
	94-B3	41	19	39
Papad	18-B2		22	47
	52-B2	41	36	55
	57-B2	41	26	39
	57-B3	42	30	41
	57-B5			23
	70-B1	42	28	41
	93-B2			12
	93-B3	37		44
	113-B2			12
	113-B3	40	22	35
Wadi	2-B1	40	26	35
	6-B2	45	26	38
	49-B1	32		
	66-B3	42	30	49
	111-B1	37	13	18

[a] Others had no activity.
[b] Includes diameter of the well (5 mm).

(23%) isolates produced all the three enzymes, while 10% isolates did not produce any of the enzymes.

3.3. Thermal inactivation of sporeformers

The *D*-values were calculated from the regression analysis best-fit plot of the linear portion of the survivor curve (Fig. 1). In glucose-supplemented brain–heart infusion broth, the correlation coefficient (R^2) values of decimal

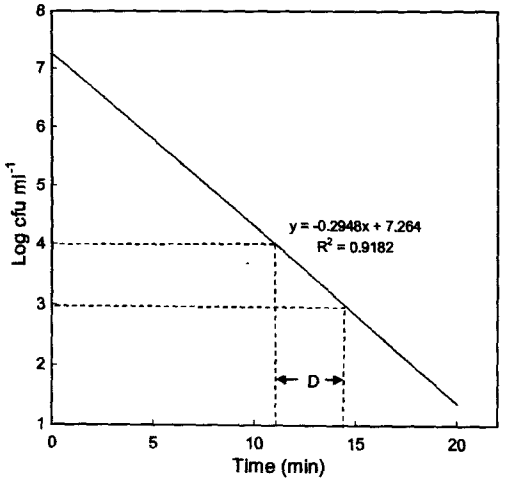

Fig. 1. Determination of *D*-value of *B. cereus* 94-B1 at 100 °C.

Table 4
Thermal inactivation of spores of *B. cereus* isolates from different food sources

Source	Isolate no.	$D_{100\,°C}$ (min)[a]
Amriti	104-B1	7.0 ± 0
	105-B1	5.2 ± 0.2
Dhokla	35-B1	5.3 ± 0.1
	37-B1	7.4 ± 0.1
Dosa	55-B1	5.6 ± 0.1
	98-B1	8.0 ± 0
Idli	94-B1	3.0 ± 0
	94-B2	4.8 ± 0.1
Papad	93-B1	9.2 ± 0.2
	1[illegible]-B1	6.2 ± 0.2
Wadi	66-B1	6.0 ± 0.1
	111-B1	6.8 ± 0.2

[a] Values are mean ± SE of triplicate determinations.

reduction plot curves for spore suspensions of 12 different isolates of *B. cereus* were at least 0.91. The mean $D_{100\,°C}$-values of 48 strains of *B. cereus* spores ranged from 3.0 to 9.2 min (Table 4).

3.4. Influence of food preservatives on growth

The effect of pH on the growth of 6 isolates, one each from the 6 different kinds of foods, is shown in Table 5. In nutrient broth, the minimum and maximum pHs permitting growth of *B. cereus* were 5.3 and 11.6, respectively.

The MICs of different preservatives on the growth of 48 strains of *B. cereus* are shown in Table 5. The growth was inhibited at 65–85 mg sodium chloride ml^{-1}. The MICs of benzoic acid and sorbic acid for growth were 0.4–0.7 mg ml^{-1} (pH 5.0–4.2) and 0.5–0.6 mg ml^{-1} (pH 5.0–4.8), respectively. Most (80%) of the tested 10 strains were resistant to 300 μg nisin ml^{-1} nutrient agar (pH 5.0).

The effect of sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 37-B1 is presented in Fig. 2. In each of these cases, the growth declined with the corresponding increase in concentration of the preservatives. These three effects along with the effect of pH were subjected to Hoke's experimental design. The selected three points were on the trend lines of growth. While the lowest limits were zero in all the cases (excepting pH), the highest limits were judiciously chosen considering the sub-inhibitory concentration levels of the preservatives along with the recommended concentrations of them. Growth of the strain against 19 different combinations of the four types of hurdles is shown in Table 6. While there was no growth in eight sets, the growth reached a maximum level in sets D and E.

3.5. Whole-cell protein fingerprinting

The WCPF of the 48 isolates yielded distinctly different band patterns (Fig. 3). Majority of the strains isolated from the same kind of food could be distinguished by their

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Table 5
pH and MIC[a] of food preservatives against the growth of *B. cereus* isolates from different food sources

Source	Target strain	pH range for growth[b]	Preservative in nutrient agar[c]			
			NaCl ($mg\,ml^{-1}$)	Benzoic acid ($\mu g\,ml^{-1}$)	Sorbic acid ($\mu g\,ml^{-1}$)	Nisin ($\mu g\,ml^{-1}$)
Amriti	104-B1	5.3–11.6	65	400 (5.0)	500 (5.0)	175
	105-B1/B2	nd	80	650 (4.3)	500	nd
	104-B3, 105-B3	nd	85	550 (4.5)	500	nd
	104-B2	nd	85	650	500	nd
Dhokla	37-B1	5.4–11.1	50	400	500	>300
	34-B1, 35-B1	nd	85	550	500	>300
Dosa	98-B1	5.3–11.6	65	450 (4.8)	500	>300
	98-B2/B3	nd	80	650	500	nd
	16-B1, 55-B1	nd	85	600 (4.4)	500	nd
Idli	94-B1	5.3–11.6	65	450	600 (4.8)	>300
	94-B2	nd	85	450	600	nd
	94-B3	nd	85	600	500	nd
Papad	113-B1	5.3–11.6	70	400	500	>300
	57-B5	nd	80	450	500	nd
	113-B3	nd	85	550	500	nd
	18-B2/B3/B5, 52-B2, 57-B2/B3/B4, 70-B1/B2, 93-B1/B2/B3, 113-B2	nd	85	600	500	nd
	52-B1	nd	85	650	500	>300
Wadi	111-B1	5.3–11.6	70	450	500	175
	111-B2	nd	70	450	500	nd
	111-B3	nd	80	550	500	nd
	6-B2, 49-B2	nd	85	550	500	nd
	49-B1	nd	85	550	500	>300
	2-B3, 66-B2/B3/B4/B5	nd	85	600	500	nd
	46-B2	nd	85	650	500	>300
	66-B1	nd	85	650	500	nd
	2-B1	nd	85	700 (4.2)	500	nd

[a] MIC (minimum inhibitory concentration) signified minimum concentration of the preservative at which growth was completely inhibited.
[b] nd, not determined.
[c] Values within parentheses indicate pHs of media after the additions.

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WCPF patterns. Fig. 4 shows a simplified version of the dendrogram obtained. Basically, the profiles could be grouped into four major clusters emerging at a similarity level of 60%. These clusters, designated A through D, represented 40%, 50%, 6% and 2%, respectively, of the total strains.

4. Discussion

Antibiotic sensitivity studies shows that all the *B. cereus* isolates were multi-drug resistant; each of these was resistant to at least nine different antibiotics. Most of the antibiotics against which the isolates showed resistance belonged to different groups, including β-lactam (ampicillin, carbenicillin, cephalothin, cloxacillin and penicillin G), glycopeptide (vancomycin), peptide (bacitracin and polymyxin B) and trmethoprim. Most of these antibiotics inhibit synthesis of prokaryotic cell wall. As expected, metronidazole, an antiprotozoal drug, had no action on any of the isolates. All these isolates, enriched on *B. cereus* selective medium (which contained 100 U polymyxin B ml^{-1}), were resistant against even a higher concentration (300 U $disc^{-1}$) of polymyxin B. However, an earlier study (Banerjee & Sarkar, 2004) reported susceptibility of only 8% of the 84 *B. cereus* isolates from spices to this higher concentration of polymyxin B. Out of the 48 isolates, 10% were resistant against 9 antibiotics, 21% against 10 antibiotics, 38% against 11 antibiotics, 29% against 12 antibiotics, and 2% against 13 antibiotics. The presence of such a high number of multiple-antibiotic resistant strains of *B. cereus* in foods is a matter of concern. Although use of antibiotics is not the rule of treating gastroenteritis, it is a common therapeutic measure taken (e.g., vancomycin) to combat acute necrotizing gastritis caused by *B. cereus*, particularly in immunocompromised patients (Le Scanff et al., 2006). Genes for resistance and molecular transfer mechanisms have been shown to be the same in bacteria from food and from pathogenic (animal and human) samples. This situation led to scientific and political efforts to handle the problems of antibiotic resistance in food (Teuber, Meile, & Schwarz, 1999).

Microbial enzymes are major causes of quality deterioration and food spoilage (Braun et al., 1999). Activity of the enzymes, like protease, lipase and amylase indicates spoilage potentiality of the producing organisms. While 50% of the 48 isolates were capable of producing at least

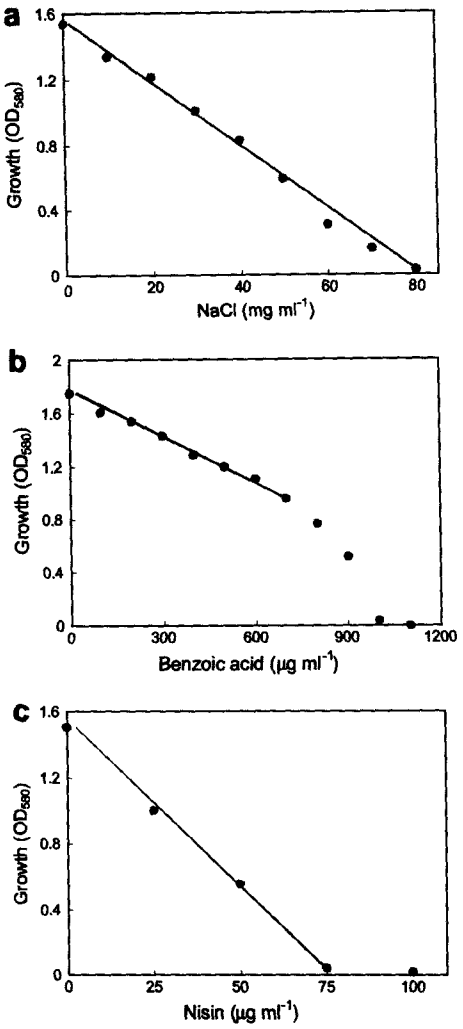

Fig. 2. The effect of sodium chloride (a), benzoic acid (b) and nisin (c) concentration on the growth of *B. cereus* 37-B1.

one of these three enzymes, 23% could produce all these enzymes in vitro. From the results obtained, it can be concluded that many of the strains of *B. cereus* present in fermented foods have the potentiality of causing food spoilage also.

The $D_{100\,°C}$-values of the spore suspensions of 12 different *B. cereus* isolates (two from each of the 6 kinds of foods) were 3.0–9.2 min, which suggest that time–temperature exposure at an appropriate level during cooking may destroy heat-sensitive spores, but not the heat-resistant ones. However, the response of *B. cereus* spores to heating is strain-dependent and is influenced by medium composition (Chung & Sun, 1986). $D_{100\,°C}$-values of 2.7–3.1 min in skimmed milk (Mikolajcik, 1970), 0.6–27.0 min in demineralized water (Rajkowski & Mikolajcik, 1987) and 3.5–5.9 min in BHIG (Banerjee & Sarkar, 2004) were reported for *B. cereus* spores. Spores of *B. cereus*, which may survive heat treatment (e.g., cooking, steaming and frying) during final preparation of these foods, germinate when kept at room temperature before consumption. The data represented here can be used as an aid to predict the time required at 100 °C to achieve a certain number of log-cycle reductions of this potentially dreadful sporeformer.

Now-a-days the use of natural antimicrobial compounds to preserve foods are widely used because of the consumers' demand for additive-free, fresher and more natural tasting food products, while maintaining microbiological safety (Gould, 1996). The addition of salt to foods has been known for centuries. As the common salt acts mainly by reducing water activity (a_w) of foods, its spectrum of action is governed by the demands imposed on a_w by the various microorganisms (Lueck, 1980). In this study, 73% of the strains tolerated a high salt concentration (>80 mg ml^{-1}), which supports halotolerant character of the bacterium.

In most cases weak organic acids, like benzoic acid, have been permitted for food preservation for many years. Benzoic acid is used as an acid or sodium salt at a concentration of 0.5–2.0 mg ml^{-1} in many low pH products (Ray, 2001). In this study, all the isolates were inhibited by benzoic acid concentration within the permissible range. Sorbic acid and sorbates are permitted in all countries of the world for the preservation of many foods, like margarine, cheese, dried and bakery foods using 0.5–2 mg g^{-1} (Ray, 2001). Sorbic acid is a GRAS (generally recognized as safe) substance and its use is permitted in any food product to which preservatives may be added (Lueck, 1980). In this study all the tested strains were inhibited at 0.6 mg sorbic acid ml^{-1}, the level which is lower than the one (0.9 mg ml^{-1}) reported by Del Torre, Della Corte, and Stecchini (2001). As the undissociated form of benzoic and sorbic acids is primarily responsible for antimicrobial activity, and it is highly pH dependent (Jay, 1996), they would be much effective in controlling pathogenic bacteria in lactic fermented foods. As all the *B. cereus* strains tested were inhibited by the acids in their permissible concentration, these acids can be used effectively in controlling the pathogen. Although nisin exhibits a wide range of inhibitory effects against Gram positive sporeformers and pathogens (Hurst, 1981), in the present study it had a low level of inhibitory activity ($\geqslant$175 μg ml^{-1}) against the strains tested. This result was consistent with the finding of Banerjee and Sarkar (2004) who have reported MICs of $\geqslant$125 μg ml^{-1} for the *B. cereus* strains tested. The use of nisin as the sole preservative for a product would probably be unwise, as multiple exposures of a pathogen to nisin would greatly increase the probability of generating stable resistant mutants. However, coupling nisin with several other common food preservation strategies greatly reduces the frequency at which resistance arises (De Martinis, Crandall, Mazzotta, & Montville, 1997). In fact, in most applications, nisin serves as one part of a multiple-barrier inhibitory system.

Prior to undertaking the in vitro multiple-hurdle preservation strategy, the effects of individual hurdles, namely pH, sodium chloride, benzoic acid and nisin on the growth of one isolate were studied separately. Out of 19 different combinations, there was no growth in eight sets. The

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Table 6
Growth of *B. cereus* 37-B1 in nutrient broth as influenced by a combination of four independent variables (hurdles) following Hoke's response surface design

Hurdle combination no.	Independent variables				Growth (OD_{580}) Mean ± SE[a]
	pH	Sodium chloride (mg ml^{-1})	Benzoic acid (μg ml^{-1})	Nisin (μg ml^{-1})	
A	5.6	20	300	25	0
B	6.4	0	300	25	0.32b ± 0.03
C	6.4	20	0	25	0.18d ± 0.02
D	6.4	20	300	0	.43a ± 0.02
E	5.6	0	0	0	.46a ± 0.04
F	5.6	40	600	50	0
G	7.2	0	600	50	0.18d ± 0.01
H	7.2	40	0	50	0
I	7.2	40	600	0	0.25c ± 0.02
J	7.2	40	0	0	0.26c ± 0.01
K	7.2	0	600	0	0.30b ± 0.02
L	7.2	0	0	50	0.24c ± 0.03
M	5.6	40	600	0	0.21d ± 0.01
N	5.6	40	0	50	0
O	5.6	0	600	50	0.26c ± 0.02
P	6.4	40	600	50	0
Q	7.2	40	600	50	0
R	7.2	40	300	50	0
S	7.2	40	600	25	0

[a] Values with standard error (SE) were obtained from three replicates. Means within the column sharing a common letter are not significantly different ($P < 0.05$).

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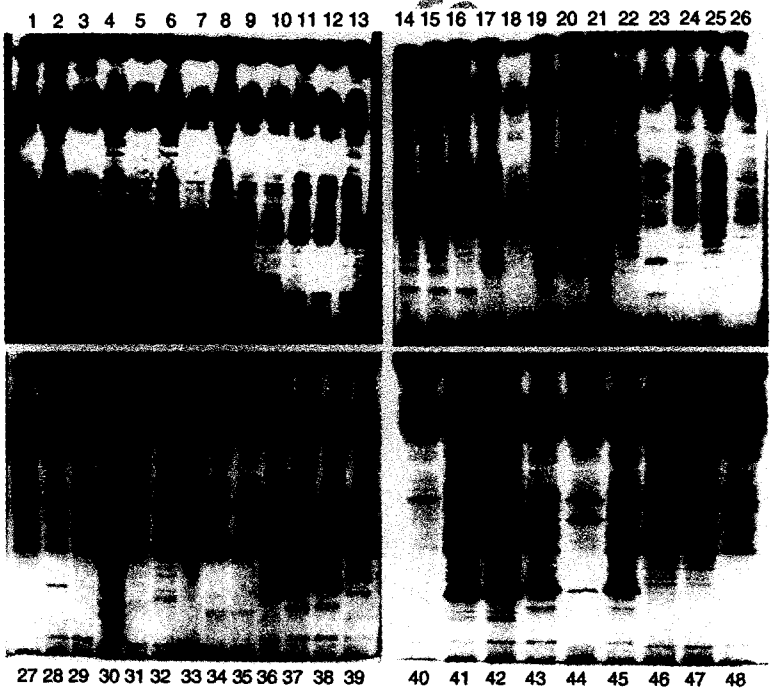

Fig. 3. SDS–PAGE profiles of whole-cell *B. cereus* strains. Lanes: 1, 111-B3 (A11); 2, 111-B2 (A11); 3, 111-B1 (A11); 4, 66-B4 (A9); 5, 66-B3 (C2); 6, 66-B2 (A9); 7, 66-B1 (C1); 8, 49-B2 (A5); 9, 49-B1 (A4); 10, 46-B2 (A4); 11, 6-B2 (A2); 12, 2-B3 (A3); 13, 2-B1 (A1); 14, 18-B2 (B1); 15, 18-B3 (B2); 16, 18-B5 (B3); 17, 52-B1 (B5); 18, 52-B2 (B14); 19, 57-B2 (B6); 20, 57-B3 (B6); 21, 57-B4 (B6); 22, 57-B5 (B15); 23, 70-B1 (B17); 24, 70-B2 (B7); 25, 93-B1 (B16); 26, 93-B2 (B16); 27, 93-B3 (B10); 28, 113-B1 (A12); 29, 113-B2 (B11); 30, 113-B3 (B4); 31, 34-B1 (B11); 32, 35-B1 (C3); 33, 37-B1 (B12); 34, 16-B1 (D); 35, 55-B1 (D); 36, 98-B1 (A7); 37, 98-B2 (A10); 38, 98-B3 (A10); 39, 66-B5 (A8); 40, 104-B1 (B8); 41, 104-B2 (B8); 42, 104-B3 (B8); 43, 105-B1 (A6); 44, 105-B2 (A13); 45, 105-B3 (A5); 46, 94-B1 (B9); 47, 94-B2 (B9); 48, 94-B3 (B13). Cluster/ subcluster numbers are shown within parentheses.

judicious combination found for the cessation of growth of *B. cereus* 37-B1 was 20 mg sodium chloride ml^{-1}, 300 μg benzoic acid and 25 μg nisin ml^{-1} at pH 5.6 (set A), in which all the three preservatives were in moderate concentrations. The same effect was achieved by the omission of benzoic acid, however only at higher concentrations of the

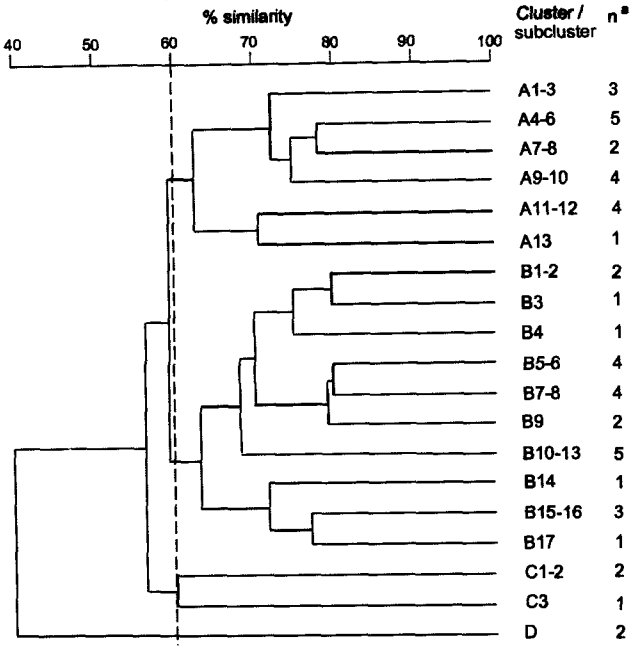

Fig. 4. Simplified dendrogram based on the UPGMA clustering of similarity coefficients (S_D) of whole-cell protein profiles of the 48 strains of *B. cereus* (as shown in Fig. 3). The fingerprint patterns were grouped into four major clusters, designated A through D, on the basis of ⩾60% similarity (arbitrarily chosen) among the strains used. [a] n, number of strains in cluster/subcluster.

other two preservatives (sets H and N). Harris, Fleming, and Klaenhammer (1991) found that the effectiveness of nisin was slightly enhanced by supplementing 25 mg sodium chloride ml^{-1} in a nisin solution. The results of this study will be the basis for an efficient application of hurdle technology in preserving such legume-based fermented foods.

On the basis of WCPF analysis, the 48 isolates of *B. cereus* were found to belong to 34 subclusters. All these subclusters, excepting two (A5 and B11) were source (food)-wise homogeneous. The isolates from wadi were restricted to clusters A (12 isolates out of 14 grouped under 'A') and C (2/14) only, and those from papad were confined to clusters A (1/17) and B (16/17). Cluster D contained isolates from dosa only. All the three isolates from idli were confined to cluster B. This study reveals a diversity of the organism at subspecies level and a relative preference of the WCPF subclusters to particular types of legume-based fermented foods.

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