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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 $D_{100\,^{\circ}C}$-values for 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 $\geqslant$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.

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 used, 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* isolates 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