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Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala)

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Abstract

A total of 126 isolates of *Bacillus* and related genera from indigenous, spontaneously fermented soybeans (Kinema) and locust beans (Soumbala) were characterized with the purpose of defining interspecific, as well as intraspecific relationships among the components of their microflora. *B. subtilis* was the dominant species, and species diversity was more pronounced in Soumbala than in Kinema. While from Kinema, six species were isolated (*B. subtilis*, *B. licheniformis*, *B. cereus*, *B. circulans*, *B. thuringiensis* and *B. sphaericus*), in Soumbala, the species found were *B. subtilis*, *B. thuringiensis*, *B. licheniformis*, *B. cereus*, *B. badius*, *Paenibacillus alvei*, *B. firmus*, *P. larvae*, *Brevibacillus laterosporus*, *B. megaterium*, *B. mycoides* and *B. sphaericus*. Genomic diversity in the isolates of *B. subtilis* was investigated by random amplified polymorphic DNA (RAPD) analysis using the polymerase chain reaction (PCR). The RAPD-PCR fingerprint analysis showed a high level of diversity. With more than 90% similarity, all 52 RAPD subdivisions were source and continent-wise homogeneous. Profiles of carbon source fermentation also showed a wide but corresponding phenotypic diversity, largely corresponding with RAPD subdivisions. The various strains were tested for several criteria for functionality in soybean fermentation, viz. protein degradation, pH increase, and development of desirable stickiness caused by viscous exopolymers. Profiles of functionality, based upon estimations of pH, free amino nitrogen and stickiness were associated with genotypic and phenotypic profiles. Notwithstanding the heterogenous fermentation results for some genotypic profiles, a ranking of RAPD groups is possible and can be useful in the further selection and study of *B. subtilis* strains.

Keywords: *Bacillus subtilis*; Kinema; Soumbala; PCR; RAPD; DNA fingerprinting; Soybean fermentation; pH; Amino nitrogen; Stickiness

1. Introduction

Bacillus subtilis is an important starter culture for alkaline food fermentations. It is found predominantly in the production of certain important traditional fermented foods, such as Indian Kinema, Chinese Dou-shi, Thai Thua-nao, Japanese Natto and West African Soumbala. By hydrolyzing proteins to peptides and amino acids and releasing ammonia, the alkaline pH helps *B. subtilis* to dominate the fermentation by creating an inhibitory environment towards spoilage microorganisms. Alkaline fermentations also yield amino acid–peptide mixtures that are closely related to meat/protein food replacements as they

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provide the same amino acids for nutrition that meats provide following digestion and they also have a meat-like flavour, which contributes to food enjoyment (Steinkraus, 1995).

To make traditional Kinema, soybeans are washed, soaked overnight in water, boiled in fresh water until soft and crushed lightly to dehull. The grits are wrapped with locally available leaves and sackcloth and left to ferment for 1–2 days in a warm place (25–30 °C) until the beans are covered with a stringy, mucilaginous coating and a typical Kinema flavour dominated by pyrazines is developed (Owens et al., 1997). Addition of a small amount of firewood ash to the crushed beans is optional. Kinema (Fig. 1a) is used to prepare a thick curry, eaten along with rice (Tamang et al., 1988).

In many of the West African countries, a fermented product of seeds from the African locust bean tree (*Parkia biglobosa*) is widely used as a condiment for preparing soups and stews. It is called Soumbala in

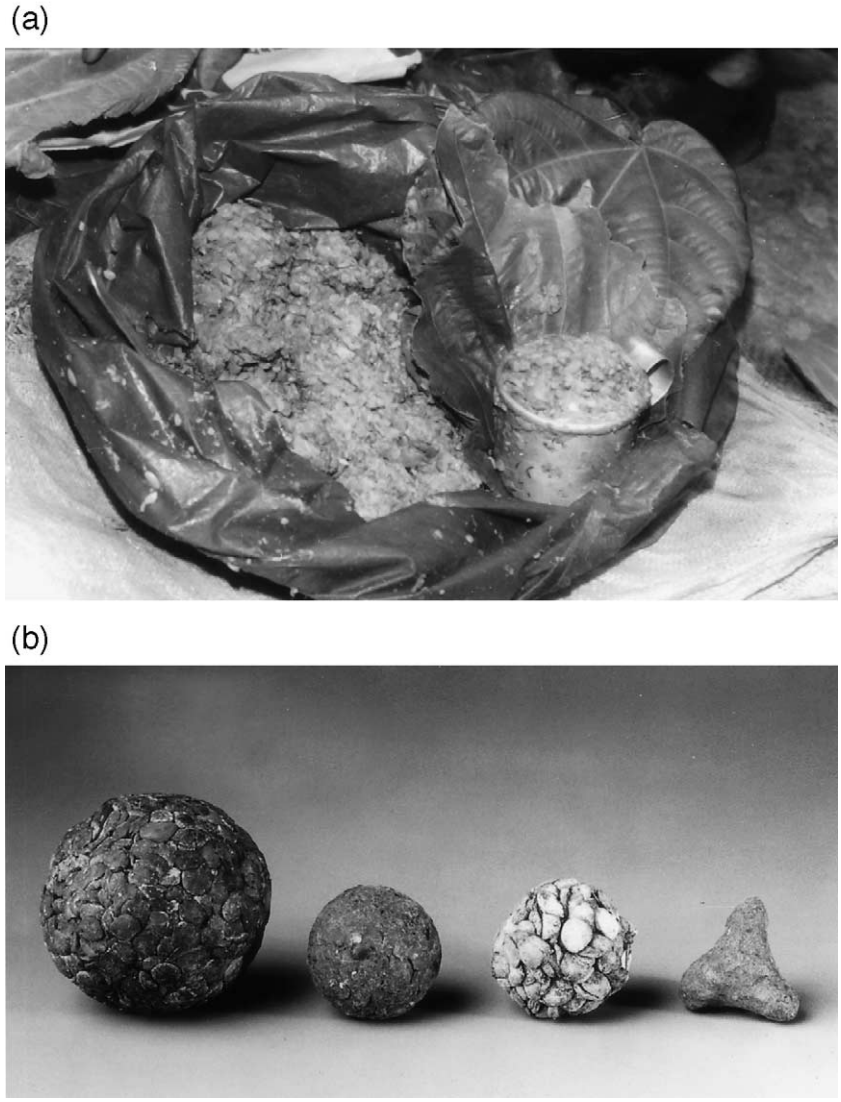

Fig. 1. Kinema (a) and Soumbala (b) samples.

Burkina Faso and Mali, Soumbara in Ivory Coast, Nététou in Senegal, Dawadawa or Daddawa in Northern Nigeria, and Iru, Dorowa or Ogin-igala in the Savannah areas of Nigeria. The seeds are washed and boiled in water in a covered container for 18–24 h with occasional renewal of water to swell the seeds and soften the very tough seed coats which are then removed by pounding and flushing. The heap (10–15 cm thick) of cotyledons in a calabash is then covered with leaves and sackcloth and left to ferment for 3–4 days at ambient temperature (25–35 °C) when the beans become covered in a sticky mucilaginous layer and develop a strong odour. The bean mass is air dried in the sun or hot shade, where the beans darken further to dark brown, and are then used loose or shaped into balls or pyramids (Fig. 1b). Wood ash is sometimes mixed to reduce the odour. To extend shelf life, salt is added in some areas (Campbell-Platt, 1987). Occasionally, soybeans also are used when locust beans are in short supply (Ogbadu and Okagbue, 1988a).

Both these fermentations are natural, i.e. no deliberate inoculation of the substrates with starter cultures is practised. It is likely that the environment and utensils become highly contaminated with a "house flora" that functions as an inoculum. Dominated by the rapid growth of the bacilli and the mucilage that is formed when the pH becomes alkaline, the invasion by other bacteria, yeasts and moulds is inhibited. Whereas *B. subtilis*, *Enterococcus faecium*, *Candida parapsilosis* and *Geotrichum candidum* are associated with Kinema (Sarkar et al., 1994), it is *B. subtilis* that has been found in all product samples in dominating numbers and that has been shown in pure culture experiments to be capable of the fermentation as a sole organism (Sarkar and Tamang, 1994; Sarkar et al., 1994; Tamang, 1999). Likewise, fermented locust bean products such as Dawadawa and Iru are dominated by *B. subtilis* in all samples (Odunfa, 1986) with concomitant presence of low numbers of opportunistic microorganisms such as *Leuconostoc* spp. (Antai and Ibrahim, 1986). *B. subtilis* has a record of safe use in vegetable protein foods (Odunfa, 1981).

Control of Kinema and Soumbala fermentations would serve the purposes of safeguarding product hygiene, as well as providing predictable organoleptic properties. A simple method was developed for the manufacture of pulverized soybean-grown pure culture starter for the Kinema process (Tamang, 1999). To enable selection of starter cultures, criteria such as proteolytic activity, pH change and formation of flavour and texture can be used (Tamang and Nikkuni, 1996). In addition, it is important to know the exact composition of the natural product microflora and be able to characterize and distinguish it at a subspecies level. The conventional methods for identification of bacteria using morphological and physiological criteria are not always sufficiently discriminatory for this purpose. A valuable technique to enhance subspecies distinction is RAPD-PCR. This method has been successfully applied to *Bacillus* as well, and is considered as a discriminatory tool for measuring diversity among strains belonging to the same species (Brousseau et al., 1993; Stephan, 1996; Stephan et al., 1994).

The objectives of this study are to reveal diversity of Gram-positive spore-forming bacteria, particularly of *B. subtilis*, occurring in market samples of two spontaneously fermented alkaline foods, Kinema and Soumbala, originating from two different continents. In addition, functional fermentation characteristics (pH, release of free amino nitrogen, stickiness) of these bacteria will be evaluated on soybeans and compared with their pattern of diversity.

2. Materials and methods

2.1. Sampling

Thirty-three samples (150–200 g) of spontaneously fermented Kinema were purchased from different retail shops in weekly markets of Gangtok, Darjeeling and Kalimpong, hill towns in West Bengal, the eastern part of India. Fourteen naturally fermented Soumbala cakes of different producers and with variable shapes were purchased at the central market of Ouagadougou, Burkina Faso. Fresh Kinema samples were collected in sterile polyethylene sampling bags, transported immediately to the laboratories in an icebox and analysed within 3 days of collection. Samples of dried Soumbala were stored refrigerated prior to analysis.

2.2. Bacterial strains

Representative 10 g portions of pasty Kinema or pulverized Soumbala were homogenized with 90 ml

sterile peptone–physiological saline (0.1% w/v neutral peptone, 0.85% w/v NaCl, pH 7.2) by the use of a Stomacher lab-blender 400 (Seward Medical, London, UK) for 1 min at 'normal' speed. Appropriate decimal dilutions (0.1 ml) of the homogenates in the same diluent were spread over plates of nutrient agar (NA; M087, HiMedia Laboratories, Mumbai, India), tryptone soya agar or plate count agar (TSA: CM131; PCA: CM 325, Oxoid, Basingstoke, Hampshire, UK) and incubated at 30 °C for 24–48 h. It was the objective to obtain as many different isolates a possible and, therefore, colonies were selected to represent the number of macroscopically different morphologies. Isolates were purified by dilution streaks on the same media. After microscopic examination of morphology, Gram-reaction and endospore formation, purified cultures were grown on slants of the same media and stored at 4 °C.

The following isolates were obtained. From Kinema (working code in brackets): samples K1 (1–3), K2 (4–6), K3 (7–9), K4 (10–12), K5 (13–15), K6 (16–18), K7 (19–21), K8 (22, 23), K9 (24–26), K10 (27, 28), K11 (29–31), K12 (32–34), K13 (35, 36), K14 (37), K15 (38–40), K16 (41–43), K17 (44–46), K18 (47), K19 (48–50), K20 (51, 52), K22 (53–55), K23 (56, 57), K24 (58, 59), K25 (60, 62), K26 (63–65), K27 (66–68), K28 (69–71), K29 (72), K30 (73–75), K31 (76–78), K32 (79), K33 (80), K34 (81, 82). From Soumbala: samples S1 (90–93, 94, 95), S2 (96–100, 101, 102), S3 (103–106), SA (107–109), SB1 (110), SB2 (111–113, 135), SC (114, 115), SD (116), SE (117, 132), SF (118, 119), SG (120–123, 134), SH (124–126), SI (131, 133), SJ (127–130). The following strains were used for comparative purposes: *B. subtilis* (*natto*) (code 61) "Miura" of Miyagino Natto Starter, Sendai, Japan, kindly provided by Dr. J.P. Tamang, Sikkim Government College, Gangtok, India; *B. subtilis* (code 83) DKW1 (MTCC–1747 Microbial Type Culture Collection, Chandigarh, India), *B. subtilis* (code 87) (ATCC–6633 American Type Culture Collection, Rockville, MD, USA), *B. licheniformis* (code 84) (type strain, DSM-13 Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany), *B. badius* (code 85) (type strain, DSM-23), *B. pumilus* (code 86) (type strain, DSM-27), *B. cereus* (code 88) (ATCC-12826) and (code 89) (ATCC-9139). In addition, *Brevibacillus brevis* (DSM-30) was used for primer selection.

2.3. Phenotypic characterization

Phenotypic characterization was performed according to Claus and Berkeley (1986) and Slepecky and Hemphill (1992) as summarized in Table 1. Nomenclature was updated according to Euzéby (1997).

2.4. DNA isolation

The method was based on the protocol of Mileham (1997), and the water used was demineralized and deionised. Briefly, cultures were grown on plates of TSA at 30 °C for 18–24 h. One colony of about 3 mm diameter was suspended by vortexing at high speed in 0.5 ml sterile water, contained in a sterile 1.5 ml Eppendorf tube. The suspension was centrifuged (9500*g*, 5 min) and the supernatant discarded. The cell pellet was resuspended in 100 μl InstaGene matrix (cat. no. 732-6030, Bio-Rad Laboratories, Hercules, CA, USA), incubated at 56 °C for 30 min, vortexed briefly (10 s), again incubated at 100 °C for 10 min, vortexed and centrifuged (9500*g*, 5 min). The supernatant was transferred to a new sterile Eppendorf tube and stored at −20 °C until further use.

2.5. RAPD-PCR

Based on the method described by Mileham (1997), a standard protocol was developed for amplifying reproducible RAPD fragments of *Bacillus* spp. genomic DNA. The two primers used (R1 and S1) were obtained after screening several primers of arbitrary base composition used for *Bacillus* (R1: 5′-GAAGCAGCGTGG-3′ with T_a, 35 °C) (Te Giffel et al., 1997) and *Salmonella* (S1: 5′-CCGCAGCCAA-3′ with T_a, 30 °C) (referred to as 1254 by Hilton et al., 1996). Reproducibility was tested by comparing fingerprints of duplicate DNA isolations of each type strain. The extracted DNA was amplified in a 50-μl reaction mixture consisting of a mastermix and 0.5–1.0 μg template DNA in a thin-walled 0.5-ml sterile microcentrifuge tube (cat. no. 179801, Biozym, Landgraaf, The Netherlands). The mastermix contained 10 mmol l^{-1} Tris–HCl pH 8.4, 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP (Boehringer Mannheim, Mannheim, Germany), 1.5 U *Taq* DNA polymerase (cat. no. 18038-026, Gibco-BRL, Life Technologies, Gaithersburg, MD, USA),

Table 1
Phenotypical key used for tentative identification of Gram-positive endospore forming rod-shaped bacteria[a]

(1) Allantoin or urate required	positive	*Bacillus fastidiosus*
	negative	2
(2) Catalase	positive	3
	negative	20
(3) Voges–Proskauer	positive	4
	negative	11
(4) Growth in anaerobic agar	positive	5
	negative	10
(5) Growth at 50 °C	positive	6
	negative	7
(6) Growth in 7% NaCl	positive	*Bacillus licheniformis*
	negative	*Bacillus coagulans*
(7) Acid and gas from glucose	positive	*Paenibacillus polymyxa*
	negative	8
(8) Reduction of NO_3^- to NO_2^-	positive	9
	negative	*Paenibacillus alvei*
(9) Parasporal body in sporangium	positive	*Bacillus thuringiensis*
	negative	37
(10) Hydrolysis of starch	positive	*Bacillus subtilis*
	negative	*Bacillus pumilus*
(11) Growth at 65 °C	positive	32
	negative	12
(12) Hydrolysis of starch	positive	13
	negative	17
(13) Acid and gas from glucose	positive	*Paenibacillus macerans*
	negative	14
(14) Width of rod ≥ 1.0 mm	positive	34
	negative	15
(15) Growth at pH 6.8	positive	16
	negative	*Bacillus alcalophilus*
(16) pH in VP broth <6.0	positive	28
	negative	26
(17) Growth in 10% NaCl	positive	*Bacillus pasteurii*
	negative	18
(18) Growth in anaerobic agar	positive	*Brevibacillus laterosporus*
	negative	19
(19) Acid from glucose	positive	30
	negative	24
(20) Growth at 65 °C	positive	33
	negative	21
(21) Growth in anaerobic agar	positive	22
	negative	*Bacillus azotoformans*
(22) Decomposition of casein	positive	35
	negative	23
(23) Parasporal body in sporangium	positive	*Paenibacillus popilliae*
	negative	35
(24) Growth at 50 °C	positive	*Bacillus badius*
	negative	25
(25) Growth at 5 °C	positive	*Bacillus insolitus*
	negative	*Bacillus sphaericus*
(26) Acid from arabinose	positive	*Bacillus lentus*
	negative	27
(27) Growth at 5 °C	positive	30
	negative	31
(28) Growth at 5 °C	positive	*Paenibacillus macquariensis*
	negative	29
(29) Growth in 10% NaCl	positive	*Virgibacillus pantothenticus*
	negative	*Bacillus circulans*
(30) Hydrolysis of urea	positive	*Bacillus globisporus*
	negative	*Bacillus marinus*
(31) pH in VP broth >7	positive	*Brevibacillus brevis*
	negative	*Bacillus firmus*
(32) Hydrolysis of starch	positive	33
	negative	*Bacillus schlegelii*
(33) Growth at pH 6.8	positive	*Bacillus stearothermophilus*
	negative	*Alicyclobacillus acidocaldarius*
(34) Growth in anaerobic agar	positive	*Bacillus thuringiensis*
	negative	*Bacillus megaterium*
(35) Growth in 10% NaCl	positive	*Bacillus pasteurii*
	negative	36
(36) Growth at 40 °C	positive	*Paenibacillus larvae*
	negative	*Paenibacillus lentimorbus*
(37) Colony rhizoidal	positive	*Bacillus mycoides*
	negative	38
(38) Cells motile	positive	Bacillus cereus
	negative	Bacillus anthracis

[a] Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name (based on Claus and Berkeley, 1986; Slepecky and Hemphill, 1992; Euzéby, 1997).

and 25 pmol of each primer. The mixture was overlaid with a drop of sterile mineral oil (prod. no. M5904, Sigma, St. Louis, MO, USA) and capped. In each PCR assay, a negative control without any bacterial DNA was included. PCR with random primers was carried out in a DNA thermal cycler (Perkin-Elmer 480). The temperature profile started with initial denaturation for 5 min at 94 °C, followed by 40 amplification cycles each consisting of 1 min at 94 °C, annealing for 1 min at 35° (primer R1) or 30 °C (primer S1) and extension for 2 min at 72 °C. After completion, reaction mixtures were cooled to 4 °C.

A 25-μl sample of the PCR product was mixed with 5 μl loading buffer consisting of 25% Ficoll-DL

(Sigma F9378), 0.5 g/l bromophenol blue (Sigma B5525), 0.5 g/l xylene cyanol FF (Sigma X4126) and 1.0 g/l orange G (Sigma O3756) and subjected to a submerged horizontal slab gel electrophoresis (BioZym) in a gel containing 1.6 g agarose (Seakem cat no. 50082, FMC Bioproducts, Rockland, ME, USA) in 100 ml of TBE buffer (0.089 mol l^{-1} Tris–HCl base, 0.089 mol l^{-1} boric acid, 0.002 mol l^{-1} EDTA, pH 8.0) and 0.5 μg ml^{-1} ethidium bromide (Sigma E1385) at 20 °C for 1.5 h at 90 V, so that the bromophenol blue migrated about 75% of gel length. Bands were visualized on a UV transilluminator (Chromato-Vue Transilluminator model TM20, UVP, San Gabriel, CA 91778, USA) and exposed to a Polaroid film (Polapan Pro) for 12–15 s. DNA molecular weight (0.15–2.18 kbp) marker VI (Boehringer Mannheim) was used (final concentration 1.75 μg ml^{-1}) as size standard in each run.

For maximum accuracy of comparisons, all strains was processed with the same batch of mastermix and comparisons of highly similar RAPD patterns were based on common PCR batches.

2.6. Fermentation of carbon sources

The ability to ferment various carbon sources was determined using the API system (BioMérieux, Lyon, Marcy-l'Etoile, France) according to manufacturer's instructions. The cultures were grown on plates of NA (Oxoid CM3) at 30 °C for 16–18 h. Cell material was harvested in 1 ml sterile 0.85 g l^{-1} NaCl solution which was used to inoculate 10 ml API 50 CHB medium. The tubes of 50 CH strips were filled with the inoculated medium, but the cupules were left blank. The strips were kept tilted at approximately 5°, bases of tubes uppermost, in order to trap any gas evolved, and incubated at 30 °C for 48 h. The results were read at 24 and 48 h; a test scoring positive at either reading time was considered positive.

2.7. Data analysis

The RAPD profiles (band patterns) in photographs were scanned. The data collected were normalized and further processed using the Molecular Analyst Fingerprinting v. 1.12 software (Bio-Rad) for generation of the cluster analysis in a dendrogram based on the Dice's similarity coefficient (S_D) and the unweighted pair group method using arithmetic averages (UPGMA). Interpretation of carbon source fermentation tests was carried out manually.

2.8. Laboratory-scale soybean fermentation

Soybean seeds of 'local yellow' variety, purchased from a grocery shop of a market in Siliguri town, were washed thoroughly with tap water and soaked in glass distilled water (water/beans, 4:1) for 16 h at ambient temperature (25–28 °C). After decanting soak water, the beans were lightly crushed to make grits of mainly half-cotyledons. Approximately 75 g grits and 7 ml soak water were taken into a 250-ml screw cap bottle (Schott Duran, Germany), sterilized by autoclaving (121 °C, 30 min), cooled to about 50 °C, and inoculated to a concentration of log 7–8 cells g^{-1} grits. For preparing inoculum, the test organisms were grown on nutrient agar slants at 37 °C for 24 h. The cells were scraped off with a loop, suspended in 5 ml sterile physiological saline (0.85% sodium chloride in water) and agitated for 1 min using a Vortex mixer. Cell numbers were determined using a Neubauer counting chamber and a phase contrast microscope. The bottles were loosely capped and incubated for 24 h at 45 °C and approximately 90% relative humidity in an environmental chamber.

Table 2
Tentative identification of the 126 strains from Kinema and Soumbala, according to Table 1[a]

	Kinema isolates	Soumbala isolates	Total
Paenibacillus alvei		1	1
Bacillus badius		2	2
Bacillus cereus	2	3	5
Bacillus circulans	2		2
Bacillus firmus		1	1
Paenibacillus larvae		1	1
Brevibacillus laterosporus		1	1
Bacillus licheniformis	3	3	6
Bacillus megaterium		1	1
Bacillus mycoides		1	1
Bacillus sphaericus	1	1	2
Bacillus subtilis	73	24	97
Bacillus thuringiensis	2	4	6
Total	83	43	126

[a] Data represent the number of strains tentatively identified.

2.9. Functional properties

Strains exhibiting growth to at least log 9 cfu g^{-1} grits were tested for their effect on soybean pH, release of free amino nitrogen and stickiness.

2.9.1. Determination of pH and free amino nitrogen

According to Nout et al. (1998), an accurately known weight of about 5 g fermented beans was homogenized with CO_2-free glass distilled water to a total volume of 20 ml. The pH of this suspension was measured using a conventional glass/calomel electrode. On a magnetic stirrer, the pH was subsequently increased to 8.5 using 0.1 M NaOH. After addition of 10 ml 35% formaldehyde (Merck, Mumbai, India), the suspension was stirred for 2 min, and the released protons were titrated using 0.1 M NaOH till the end-point pH 8.5 was reached. Free amino nitrogen (FAN) content was calculated on the basis of stoichiometric equivalence of one NaOH and one amino group.

2.9.2. Determination of stickiness

Samples of approximately 40 g fermented beans were stirred in a rotary movement at constant speed for 1 min on a dry metal plate, using thumb and index finger which were kept approximately 4 cm apart. After stirring, the two fingers were pressed together for 1 s and separated again. The length of the sticky threads that were pulled was measured at their break-points.

3. Results and discussion

Bacillus spp. are the organisms responsible for alkaline food fermentations and spoilage of foods in general due to their versatile metabolism and heat-resistant spores (Deák and Timár, 1988; Steinkraus, 1995). Hence, information about *Bacillus* spp. would be of primary importance in monitoring fermentation and good manufacturing practice. The lack of a complete key to the identification of *Bacillus* strains

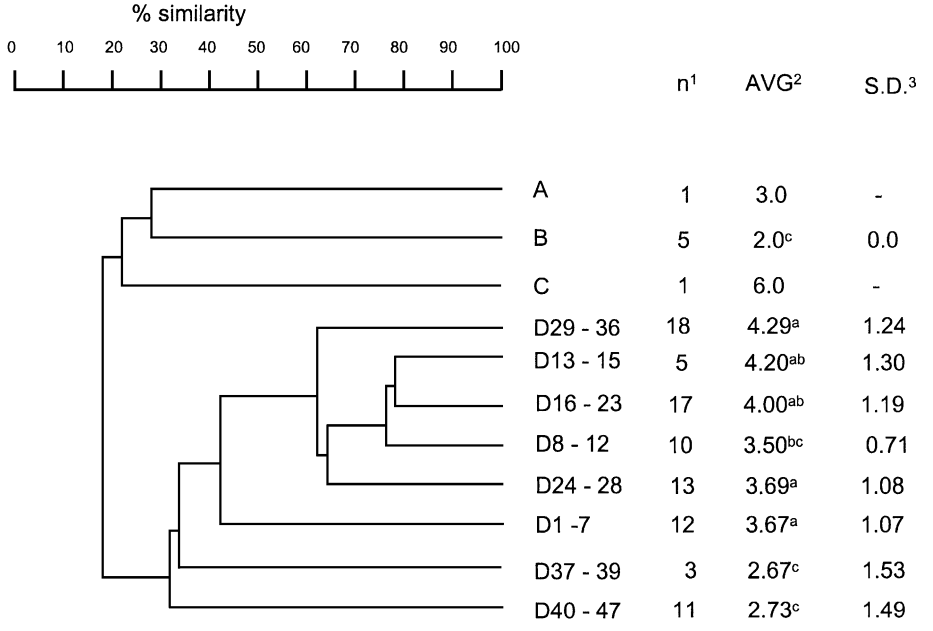

Fig. 2. Simplified dendrogram based on the UPGMA clustering of similarity coefficients (S_D) of RAPD profiles of 126 strains of *B. subtilis*, obtained by using primers R1 and S1. For each primer, a similarity matrix was created and finally joined to a matrix, in which the respective value from each primer contributed to a mean. RAPD patterns were grouped into four major clusters, designated A through D, on the basis of $\geq 30\%$ similarity (arbitrarily chosen) among the strains used. Grouped subdivisions within the predominant cluster D are shown, details of which are listed in Table 3. [1]*n* = number of strains in (sub)cluster; [2]AVG = average functional score, see also Table 3; [3]S.D. = standard deviation. Averages with the same superscript are not statistically different (unrelated two-tailed *t*-test at 5% level).

Table 3
Bacillus subtilis isolates grouping based on UPGMA clustering of similarity coefficients of RAPD profiles (combined primers R1 and S1), with data of soybean fermentation characteristics

Strain code	Origin[a]	RAPD group[b]	Selected functional characteristics			
			pH	Free amino groups (mM N/g)	Stickiness exopolymers (cm thread)	Score[c]
80	k	A	8.26	0.16	2	3
75	k	B1	8.09	0.34	1	2
17	k	B2	7.47	0.20	3	2
32			7.73	0.17	3	2
36			7.99	0.32	2	2
67	k	B3	7.85	0.37	3	2
105	s	C	8.33	0.51	8	6
108	s	D1	8.40	0.46	1	3
107	s	D2	8.53	0.71	2	5
113	s	D3	8.03	0.66	1	4
121	s	D4	8.23	0.41	2	4
99	s	D5	8.43	0.51	1	4
109			7.76	0.52	2	4
111			7.82	0.34	5	4
112			8.16	0.73	1	4
100	s	D6	8.30	0.47	0.5	3
116			7.44	0.35	1	1
122			8.11	0.54	2	5
104	s	D7	8.55	0.43	0.5	3
19	k	D8	8.20	0.45	3	4
20			7.80	0.38	3	2
21			8.01	0.46	5	4
23			8.09	0.45	2	4
24			8.00	0.48	3	4
1	k	D9	7.92	0.36	6	3
4			7.83	0.62	8	3
27	k	D10	7.94	0.46	3	3
38	k	D11	8.03	0.31	4	4
10	k	D12	7.81	0.38	4	4
30	k	D13	8.35	0.49	3	4
34			7.60	0.16	2	2
45	k	D14	8.50	0.63	3	5
35	k	D15	8.48	0.49	4	5
40			8.48	0.46	4	5
90	s	D16	ND	ND	ND	ND
93			8.70	0.51	3	5
110			8.45	0.57	6	6
3	k	D17	7.15	0.34	7	2
5			7.50	0.36	6	3
9			7.65	0.50	9	4
14			7.95	0.43	4	4
11	k	D18	8.10	0.47	4	5
13			7.78	0.39	9	3
81			8.14	0.35	6	4
25	k	D19	7.83	0.43	3	2
16	k	D20	8.05	0.39	6	4
76	k	D21	8.21	0.43	3	4
77			8.50	0.52	4	5
78			8.44	0.44	2	4
83			8.17	0.41	3	4
117	s	D22	8.13	0.41	4	4

Table 3 (*continued*)

Strain code	Origin[a]	RAPD group[b]	Selected functional characteristics			
			pH	Free amino groups (mM N/g)	Stickiness exopolymers (cm thread)	Score[c]
74	k	D23	7.83	0.34	5	5
94	s	D24	8.61	0.32	3	3
118			8.53	0.43	5	5
91	s	D25	6.70	0.08	2	1
52	k	D26	8.08	0.48	4	4
55			8.37	0.64	2	5
66			8.23	0.34	2	3
68			7.92	0.43	4	4
72			8.39	0.39	4	3
73			8.04	0.47	2	4
18	k	D27	7.64	0.37	8	3
22			7.56	0.41	5	4
33			8.39	0.43	4	4
12	k	D28	8.07	0.48	5	5
56	k	D29	8.34	0.53	2	5
57			8.17	0.58	4	6
125	s	D30	8.56	0.48	4	5
126			8.58	0.50	6	5
48	k	D31	8.22	0.56	4	6
54			8.13	0.63	2	5
63			8.20	0.48	4	5
51	k	D32	8.37	0.69	3	5
26	k	D33	7.66	0.37	7	3
39			7.81	0.23	6	3
44			7.82	0.49	9	4
46			7.60	0.42	11	4
60			8.52	0.37	0	2
49	k	D34	7.87	0.56	0	3
61	k	D35	8.44	0.63	3	5
62			7.46	0.48	5	4
64			7.79	0.50	4	3
53	k	D36	7.87	0.59	6	5
31	k	D37	8.33	0.22	2	4
65	k	D38	8.13	0.47	2	4
124	s	D39	7.19	0.31	1	1
41	k	D40	7.47	0.30	5	3
42			7.82	0.45	4	4
43			7.60	0.33	4	3
50			7.58	0.46	3	3
71	k	D41	7.91	0.28	4	2
82	k	D42	7.87	0.31	4	3
28	k	D43	7.73	0.27	1	1
7	k	D44	7.17	0.25	3	1
15	k	D45	7.65	0.39	10	3
69	k	D46	7.62	0.25	1	1
123	s	D47	8.67	0.65	9	6

See also Fig. 2 for the simplified dendrogram.

[a] k: Kinema, s: Soumbala, r: reference strain.

[b] Capitals: distinction at 30% similarity. Numbers: distinction at $\geq 90\%$ similarity.

[c] Scoring: total of scores for pH, amino-N and stickiness: pH ($<7.4=0$; $7.4-8.0=1$; $>8.0=2$), amino-N ($<0.4=0$; $0.4-0.5=1$; $>0.5=2$), stickiness ($<1=0$; $1-3=1$; $>4=2$).

prompted us to extend the dichotomous key of Slepecky and Hemphill (1992) embodying all 34 species of *Bacillus* described by Claus and Berkeley (1986), as shown in Table 1. The additional identification tests were chosen on the basis of their discriminatory power (Gower and Barnett, 1971). As our sampling was designed primarily to obtain as many different strains as possible, it is not possible to quantify the distribution of the various species in Kinema and Soumbala. Nevertheless, the number of strains of each species gives an indication of their relative abundance. *B. subtilis* had the highest occurrence in both Kinema and Soumbala (Table 2). From Kinema, *B. subtilis*, *B. licheniformis*, *B. cereus*, *B. thuringiensis*, *B. circulans* and *B. sphaericus* were isolated. The occurrence of *B. subtilis* and *B. cereus* in Kinema has been reported earlier (Sarkar et al., 1994; Nout et al., 1998). From Soumbala even more species including *B. subtilis*, *B. thuringiensis*, *B. cereus*, *B. licheniformis*, *B. badius*, *B. alvei*, *B. firmus*, *B. larvae*, *B. laterosporus*, *B. megaterium*, *B. sphaericus* and *B. mycoides* were isolated. Ogbadu and Okagbue (1988b) isolated *B. pumilus* and *B. licheniformis*, in addition to *B. subtilis*, from fermented locust beans, and *B. mycoides* from unsuccessful fermentations.

For RAPD analysis primers R1 and S1 were selected because they gave patterns for a wide range of *Bacillus* spp. reference strains, and they yielded distinctly different patterns. The majority of the isolates could be distinguished by their RAPD profiles. The isolates which could not be separated by this technique originated from the same food sample and were likely to be the same strain picked more than once during the isolation.

In order to enhance resolution between the *B. subtilis* strains, the data from RAPD patterns obtained with the two primers were combined in a single dendrogram. Fig. 2 shows a simplified version of the dendrogram obtained. Basically, the combined profiles could be grouped into four major clusters emerging at a similarity level of 30%. These clusters, designated A through D, represented 1%, 5%, 1% and 93% of the *B. subtilis* strains, respectively. Of these clusters A, B and C were homogeneous source and continent-wise. With more than 90% similarity, the clusters B and D could again be divided into three (B_1–B_3) and 47 (D_1–D_{47}) subdivisions, respectively. In Fig. 2, the branching into subdivisions is shown for the predominant cluster D. Table 3 lists the distribution of strains in subdivisions. Of these 52 subdivisions, 32 consisted of only one strain each, whereas the remaining 20 subdivisions totalling 65 strains consisted of two to seven strains each. These multi-strain subdivisions were all homogenous source and continent-wise. A similar high degree of genetic heterogeneity, using RAPD fingerprints was observed in *B. thuringiensis* (Brousseau et al., 1993) and *B. cereus* (Stephan, 1996). The wide extent of genetic diversity observed using RAPD can be partially explained by the presence of extrachromosomal plasmids larger than 20 kbp that can compete with the genomic DNA in the annealing phase, giving fragments which are amplified only in the strains harbouring that plasmid (Daffonchio et al., 1998). This indicates that strain to strain variability in RAPD profiles with a particular species can be partially explained by the presence or absence of plasmid DNA.

Twenty-one strains of *B. subtilis* were selected from six RAPD subdivisions for the evaluation of their carbon source fermentation using the API system, and to observe possible relationships between RAPD and fermentation groups. While two pairs (isolate codes 19 and 23, and 66 and 83) had identical fermentation patterns, the remaining strains had individually slightly different fermentation profiles. Of 49 carbon sources, none of the strains of *B. subtilis* was able to utilize erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, galactose, sorbose, rhamnose, dulcitol, α-methyl-D-mannoside, *N*-acetyl glucosamin, amygdalin, arbutin, salicin, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate, whereas all fermented sucrose.

On the basis of their fermentation profiles, the isolates were combined when their fermentation profiles showed maximum three different reactions (similarity $\geq 46/49$ or $\geq 94\%$). In Table 4, the relationship between RAPD subdivisions and these groups of similar fermentation profiles is shown. A good correlation was observed between RAPD subdivisions D8, D17, D21, and D27 and their fermentation profiles. On the other hand, some discrepancies occur such as isolates 99 and 122 having highly similar fermentation profiles but belonging to neighbouring RAPD subdivisions. A similar case is isolate 66 (subdivision

Table 4
Comparison of subdivisions of selected strains of *Bacillus subtilis* obtained using RAPD-PCR and fermentation profiling fingerprint approaches

Strain code	RAPD profile[a]	Fermentation of carbon sources																						
		Profile[b]	1[c]	4	5	6	11	12	13	17	18	19	21	25	27	28	29	30	32	33	34	35	36	37	40
109	D5	I	+	+	–	–	+	+	–	–	–	–	+	–	–	+	–	–	+	+	–	+	+	+	–
99	D5	II	+	–	–	–	–	+	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
122	D6	II	+	–	–	–	–	+	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–
100	D6	III	+	+	–	–	+	+	+	+	–	–	–	–	–	+	–	–	–	+	–	+	+	+	–
116	D6	IV	–	+	–	–	–	+	–	–	–	–	–	+	–	+	–	+	–	+	–	+	+	+	–
3	D17	V	+	+	–	+	+	+	+	+	–	–	+	+	–	+	–	+	+	–	–	+	+	+	+
5	D17	V	+	+	–	+	+	+	+	+	–	–	+	+	–	+	–	+	+	–	–	+	+	+	–
9	D17	V	+	+	–	–	+	+	+	+	–	–	+	+	+	+	–	+	+	–	–	+	+	+	+
14	D17	V	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	+	+	–	–	+	+	+	+
76	D21	VI	+	+	+	–	+	+	+	+	+	+	–	+	–	+	–	+	+	+	–	+	+	+	–
77	D21	VI	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	+	+	–	+	–	+	–
78	D21	VI	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	–
83	D21	VI	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	+	+	+	+
66	D26	VI	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	+	+	+	+
68	D26	VII	+	+	+	+	+	+	–	+	+	+	+	+	–	+	–	+	+	+	–	+	+	–	–
18	D27	VII	+	+	+	–	+	–	–	+	+	+	+	+	–	+	–	+	+	+	–	+	+	–	–
22	D27	VII	+	+	+	+	+	+	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	+
19	D8	VIII	+	+	–	–	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	+	+	–	+
21	D8	VIII	+	+	+	–	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	+	+	–	+
23	D8	VIII	+	+	–	–	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	+	+	–	+
24	D8	VIII	+	+	+	–	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	+

[a] Subdivisions at ≥90% similarity.
[b] Profiles with similarity ≥94%.
[c] 1—glycerol, 4—L-arabinose, 5—ribose, 6—D-xylose, 11—D-glucose, 12—D-fructose, 13—D-mannose, 17—inositol, 18—mannitol, 19—sorbitol, 21—α-methyl-D-glucoside, 25—esculin, 27—cellobiose, 28—maltose, 29—lactose, 30—melibiose, 32—trehalose, 33—inuline, 34—melezitose, 35—D-raffinose, 36—starch, 37—glycogen, 40—D-turanose.

D26) with fermentation profile, corresponding with the isolates of RAPD subdivision D21. Even though not all isolates were tested for their fermentation profile this limited analysis suggests that RAPD fingerprinting and comparison of fermentation profiles based on 50 carbon sources have discriminatory capacity of a similar order of magnitude.

The high number of different *Bacillus* strains isolated from Kinema and Soumbala confirms the diversity of the microflora and the need for effective methods for selection and control of starter cultures at subspecies level. Experimental soybean fermentations with the pure strains were carried out. All strains multiplied as expected and fermented soybeans were scored for pH, free amino-N and stickiness as shown in Table 3. Whereas some correlation ($r^2 = 0.51$) was observed between increasing pH and level of free amino-N, no correlations between pH and stickiness, or free amino-N and stickiness were evident. Of the RAPD groups containing several strains, B2, D8, D9, D15, D16, D21, D29, D30 and D33 showed a homogenous pattern of soybean fermentation. On the other hand some groups such as D5, D6, D17, D18 and D26 showed more variability. The total scores and their standard deviation (Fig. 2) illustrate these differences in homogeneity. Fig. 2 reveals that certain genotypic clusters (C, D29–D36, D13–D15, D16–D23, D24–D28 and D1–D7) gave medium to high scores whereas others (A, B, D8–D12, D37–D39 and D40–D47) performed poorly. Even though the number of strains tested for their fermentation profile is limited, it appears that groups I, II, VI and VIII (Table 4) are associated with relatively high quality scores (Table 3). Whereas this knowledge will be useful in screening and comparative studies of high-quality starter strains, final strain selection will still depend on individual performance of strains under practical conditions, also considering additional

factors such as the production of desirable volatile aroma, and competitiveness in the presence of contaminating microflora.

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