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Full Length Research Paper

Sourcing starter cultures for *Parkia biglobosa* fermentation I: Phylogenic grouping of *Bacillus* species from commercial 'iru' samples

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Twenty five isolates of *Bacillus* species were obtained from ten commercial samples of 'iru' (fermented African locust bean), after heat-treatment at 80°C for 30 min. All the isolates were Gram-positive, spore-former, catalase-positive, non-motile rods. The phylogenic relationship between the isolates was studied by repetitive PCR fingerprinting using the $(GTG)_5$ primer, referred to as $(GTG)_5$-PCR fingerprinting and 16S rRNA gene sequencing analyses. The $(GTG)_5$-PCR fingerprinting resulted in grouping of the isolates into 12 phylogenetic groups. Based on the 16S rRNA gene sequence analysis, most of the *Bacillus* isolates were found to be closely related to *Bacillus subtilis*, while strain 8B was closely related to *Bacillus licheniformis*.

Key words: 'Iru', *Bacillus*, starter culture, African locust bean, *Parkia biglobosa* fermentation, rep-PCR fingerprint, 16S rRNA sequencing.

INTRODUCTION

'Iru' is an indigenous protein-rich soup condiment produced by fermenting the cotyledons of African locust bean (*Parkia biglobosa*). It is consumed mostly by the local rural dwellers as protein supplement in the diets in many West African countries. 'Iru' is known as 'soumbala' in Burkina Faso (Ouoba et al., 2003) and 'afitin' in Benin Republic (Azokpota et al., 2006) and 'dawadawa' by the Hausa-speaking ethnic groups in West Africa (Odunfa and Adewuyi, 1985a). 'Iru' is not only consumed as a soup flavoring food additive, but also serves as a cheap meat substitute amongst poor families. A variant of 'dawadawa' is obtained by fermenting soybean (*Glycine* max) (Terlabie et al., 2006). In some Southwest Nigerian towns/ villages, 'iru' is used as a local remedy in the treatment of eye infections (Aderiye and Laleye, 2003).

The production of 'iru' being a traditional art, the fermentation is initiated by chance inoculation of natural microflora and thus the products vary considerably in quality and shelf-life. The ammonical flavor that develops in the post-fermentation product has been a major factor why the 'elites' in urban centers do not consume the product. Recently, Osho et al. (2010) conducted a comparative study on the microbial load in some local fermented foods in Nigeria, including 'iru'.

Microorganisms associated with the fermentation are mostly strains of *Bacillus subtilis* group, while *Lactobacillus* sp. and *Staphylococcus epidermidis* are present in lower numbers (Odunfa, 1981a). The optimum conditions (temperature and time) required for fermentation are 35°C and 36 h respectively (Odunfa and Adewuyi, 1985a). Starter culture experiments have proved that strains belonging to the *B. subtilis* group are responsible for the fermentation (Odunfa and Adewuyi,

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Table 1. Sources of commercial 'iru' samples in Southwest Nigeria.

S/No.	Place of sample collection	Sample code
1	Iloro-Ekiti (Ekiti State)	S1
2	Oye-Ekiti (Ekiti State	S2
3	Isan-Ekiti (Ekiti State)	S3
4	Ado-Ekiti (Ekiti State)	S4
5	Ikare-Akoko 1 (Ondo State)	S5
6	Ikare-Akoko 2 (Ondo State)	S6
7	Ikare-Akoko 3 (Ondo State)	S7
8	Rore (Kwara State)	S8
9	Iru-pete (Lagos State)	S9
10	Ikare-Akoko 3 (Ondo State)	S10

1985b). The strains of *Bacillus species* (*B. subtilis, Bacillus licheniformis* and *Bacillus pumilus*) differed in their growth and extracellular enzymes production in broth medium (Aderibigbe and Odunfa, 1990). Some indigenous fermented foods have enjoyed technological advancement and standardization by the use of proven strains as starter cultures. These include soy sauce, natto and tempeh. A key factor in industrialization of 'iru' production is the development of proven strains of bacteria as starter cultures for the fermentation (Latunde-Dada, 1995). This will ensure standardization of product, having longer shelf-life and high hygienic quality. Hence, the purpose of this study was to isolate strains of *B. subtilis* group from commercial samples of 'iru', confirm their phylogenic relationship, screen them through starter culture experiments and evolve a new strains which have the potentials of being developed into starter cultures for industrial scale production of 'iru'. This paper reports on the isolation and characterization of strains of *Bacillus* species in commercial 'iru' samples.

MATERIALS AND METHODS

Sources of 'iru' samples

'Iru' samples were bought from reputable local vendors in Southwest Nigeria, whose products are judged as of good quality (by visual observation and perception of ammonia odor threshold). The place from where the samples were collected and codes given to them are shown in Table 1. The samples were kept in sterile 50 ml polystyrene bottles and stored at -20°C until their analysis.

Culturing, Isolation and identification of bacterial isolates from Iru samples

One gram of Iru sample was weighed and transferred into 9 ml of sterile 0.1% peptone water, mixed by vortexing and heated in a water bath at 80°C for 30 min. Microbes were isolated by means of the serial dilution plating on nutrient agar medium (NA, Difco) containing 0.3% (w/v) beef extract, 0.5% (w/v) peptone and 1.5% (w/v) agar, pH 7.2. The plates were incubated at 35°C for 18 h. A pure culture was obtained by repeated transfers of individual colonies on nutrient agar medium as mentioned previously. Number of colonies and their characteristics were recorded. The standard methods of Gram-staining, motility test (wet-mount) and catalase test were performed. The modified method described by Hamouda et al. (2002) was used to identify the spore formers. The reference strains used were *Bacillus amyloliquefaciens* KCTC 1660^T, *B. subtilis subsp. subtilis* KCTC 3135^T, *Bacillus vallismortis* KCTC 3707^T, *Bacillus licheniformis* KCTC 1918^T, and *Bacillus mojavensis* KCTC 3706^T. Unless otherwise stated, strains were grown in the same medium and cultivated at 37°C for 24 h.

Taxonomic grouping of *Bacillus* isolates through molecular methods

Extraction and purification of DNA

A single colony was inoculated into 5 ml nutrient broth (NB) and incubated overnight at 35°C. The starter culture (5 ml) was used to inoculate 50 ml NB in 250 ml conical flask and incubated with shaking at 200 rpm at 35°C overnight. Cultures were centrifuged at 10,000 rpm (4°C) for 10 min and pellets were re-suspended in 5 ml of NB. A 1.3 ml of cells' suspension was added into a 1.5 ml microtube and centrifuged at 11,000 rpm (4°C) for 1min. Total genomic DNA was extracted by using a Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions, with minor modification by Plengvidhya et al. (2004). In this modification, the cell lysis solution contains 5 µl of mutanolysin (2500 U/ml; Sigma, St. Louis, MO, USA) in addition to 10 µl of lysozyme (10 mg/ml, Sigma, USA). The DNA quality was determined by electrophoresis on 1% agarose gel. After electrophoresis, the gels were stained in 0.5X TAE containing 0.5 µg/ml of ethidium bromide (Fluka) for 15 min. Pictures of the gels were digitally captured using the Bioimaging System GeneGenius (SynGene, Cambridge, England). The DNA concentration was measured at 260 nm with spectrophotometer. For rep-PCR, the DNA was adjusted to 50 ng/µl with TE buffer.

Rep-PCR genomic fingerprinting

DNA extracted from selected *Bacillus* isolates were subjected to rep-PCR analysis using primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3') as described by Sumpavapol et al. (2010). Amplicons were separated on 1% LE Seakem® agarose (BME, Rockland, ME, U.S.A) in 0.5X TBE at 120 V for 2 h and 40 min (2 x 1 h: 20 min) prior to stain in 0.5X TBE containing 5 µg/ml ethidium bromide

Table 2. Microbial load and cultural characteristics of *Bacillus* species in commercial 'iru' samples.

Sample Code	Microbial load (x 10^7 cfu/g)	Cultural characteristics of bacterial isolates on NA					
		Isolate code	Color	Margin/edge	Elevation	Surface	Size
S1	21	1A	Cream	Lobate	Flat	Dry	++++
		1B	"	"	"	"	++++
S2	37	2A	"	"	"	"	++++
		2B	"	"	"	"	++++
S3	7.5	3A	"	Lobate/Rhizoid	"	"	+++
		3A-2	"	Lobate/Rhizoid	"	"	+++
		3B	"	Lobate	"	"	++++
S4	42	4A	"	Rhizoid	"	"	+++
		4B	Cream/White	"	"	"	++++
S5	8.5	5A	Cream	"	"	"	+++
		5B	"	"	"	"	+++
S6	24	6A	"	"	"	"	+++
		6B	"	"	"	"	+++
S7	29.5	7A	Cream	Lobate	Flat	Dry	+++
		7B	"	Rhizoid	"	Dry/Glossy	+++
S8	9.0	8A	"	"	"	"	++++
		8B	"	Entire	Raised/ Flattened	Glossy	++++
		8B-1	"	Rhizoid	"	"	++++
		8C	"	Lobate/Rhizoid	Flat	"	++++
S9	48	9A	"	"	"	Dry	++++
		9B	"	"	"	"	++++
		9C	"	Lobate	"	"	++++
S10	11	10A	"	Lobate/Rhizoid	"	"	++++
		10B	"	Rhizoid	"	"	+++
		10C	"	"	"	"	++++

(Sigma, USA) for 10 min and destained in tap water for 20 min, with shaking (UMAC OMRON H7ER Orbital shaker, 28 rpm), The gel image was captured by using an image scanner Typhoon 9410 (Amersham Biosciences). The DNA patterns were analyzed by using a pattern analysis software package, Gel Compar II, Version 4.5 (Applied Math, Belgium). Pearson product-moment correlation coefficient was used to calculate similarities between patterns and a dendogram was obtained by means of unweighted pair group method with arithmetic average (UPGMA).

16S rRNA gene sequence analysis

16S rRNA gene sequencing was carried out using the methods reported by Ruiz-Garćia et al. (2005). The 16S rRNA gene was amplified by PCR with universal bacterial primers, 16S-27 F (5'-AGAGTTTGATCATGGCTCAG-3') annealed at positions 8 to 27 and 16S-1488 R (5'-CGGTTACCTGTTAGGACTTCACC-3') annealed at positions 1511-1488 (*E. coli* numbering) according to Brosius et al. (1978). Amplification reaction was carried out in 25 µl volume, using the Takara *Ex Taq* DNA polymerase and buffer system (Takara Mirus Bio Corporation, Madison, WI). The final PCR mixture comprised 1X *Ex Taq* buffer (with 1.5 mM $MgCl_2$), a 200 µM concentration of each deoxynucleoside triphosphate, an 0.2 µM concentration of each primer, 1 unit of *Ex Taq* DNA polymerase, and 50 ng of template DNA. Amplification was carried out in a thermocycler (GeneAmp PCR System 2400, PE Biosystems, Foster, California, U.S.A.) with the following cycling program: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing at 55°C for 15 s, and extension at 72°C for 90 s, and a final extension step at 72°C for 5 min. The PCR product was purified using QIAquick-PCR purification kit (Qiagen). The double-stranded DNA was sequenced with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster,

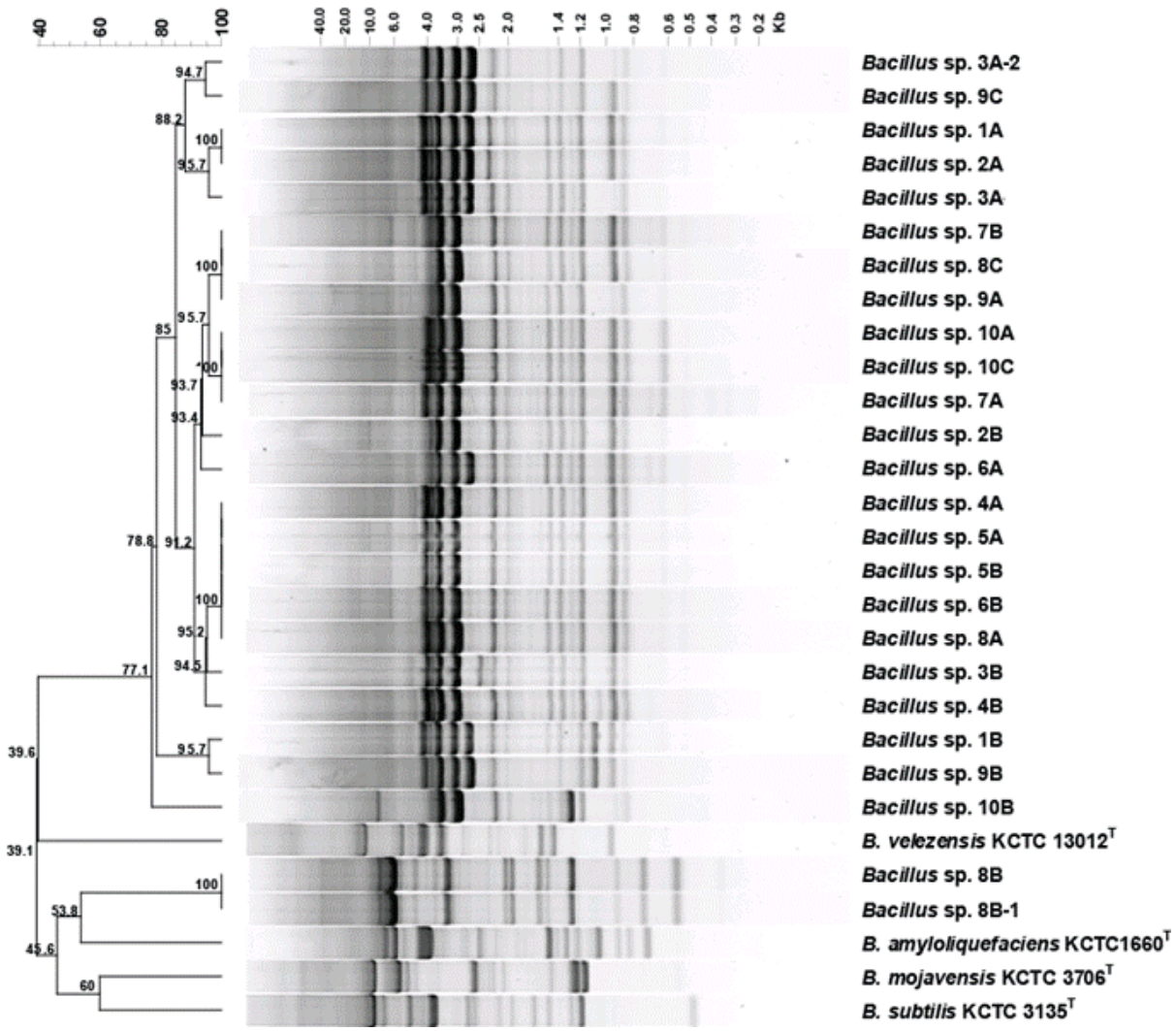

Figure 1. Illustrations of the cluster analysis and $(GTG)_5$-PCR genomic fingerprint of *Bacillus* sp. isolated from Iru and related species of the genus *Bacillus* species. Dendrogram was based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm.

California, U.S.A.) according to the manufacturer's instruction, by the use of the following four primers; 16S-27 F (5'-AGAGTTTGATCATGGCTCAG-3') annealed at positions 8 to 27, 16S-421 R (5'-CGGATCGTAAAGCTCTGTTG-3') annealed at positions 401 to 421, and 16S-1488 R (5'-CGGTTACCTGTTAGGACTTCACC-3') annealed at positions 1511-1488. The PCR products were sequenced with an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, Foster, California, U.S.A.). The 16S rRNA gene sequences were aligned along with the selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the program CLUSTAL_X (version 1.81) (Thompson et al., 1997). Gaps and ambiguous bases were eliminated from the calculations. The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with a program MEGA (version 2.1) (Kumar et al., 2001). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 samplings.

RESULTS AND DISCUSSION

Table 2 shows the microbial load and the cultural characteristics of the isolates obtained from 10 commercial 'iru' samples. All the *Bacillus isolates* were Gram- positive, spore-forming rods, catalase positive, and most were non-motile. 'Iru' produced by commercial vendors vary considerably in sensory qualities. Odunfa and Adewuyi (1985b) reported that strains of *Bacillus* involved in fermentation could influence the quality of the product. Many strains were obtained from 'iru' samples (Odunfa and Oyewole, 1986); which varied in growth rate and extracellular proteinase, amylase, polygalacturonase, galactanase and sucrase production in NB medium (Aderibigbe and Odunfa, 1990). Species of *Bacillus* have been reported to be involved in the fermentation of other plant seeds in the production of natto, 'thua-nao',' ugba',

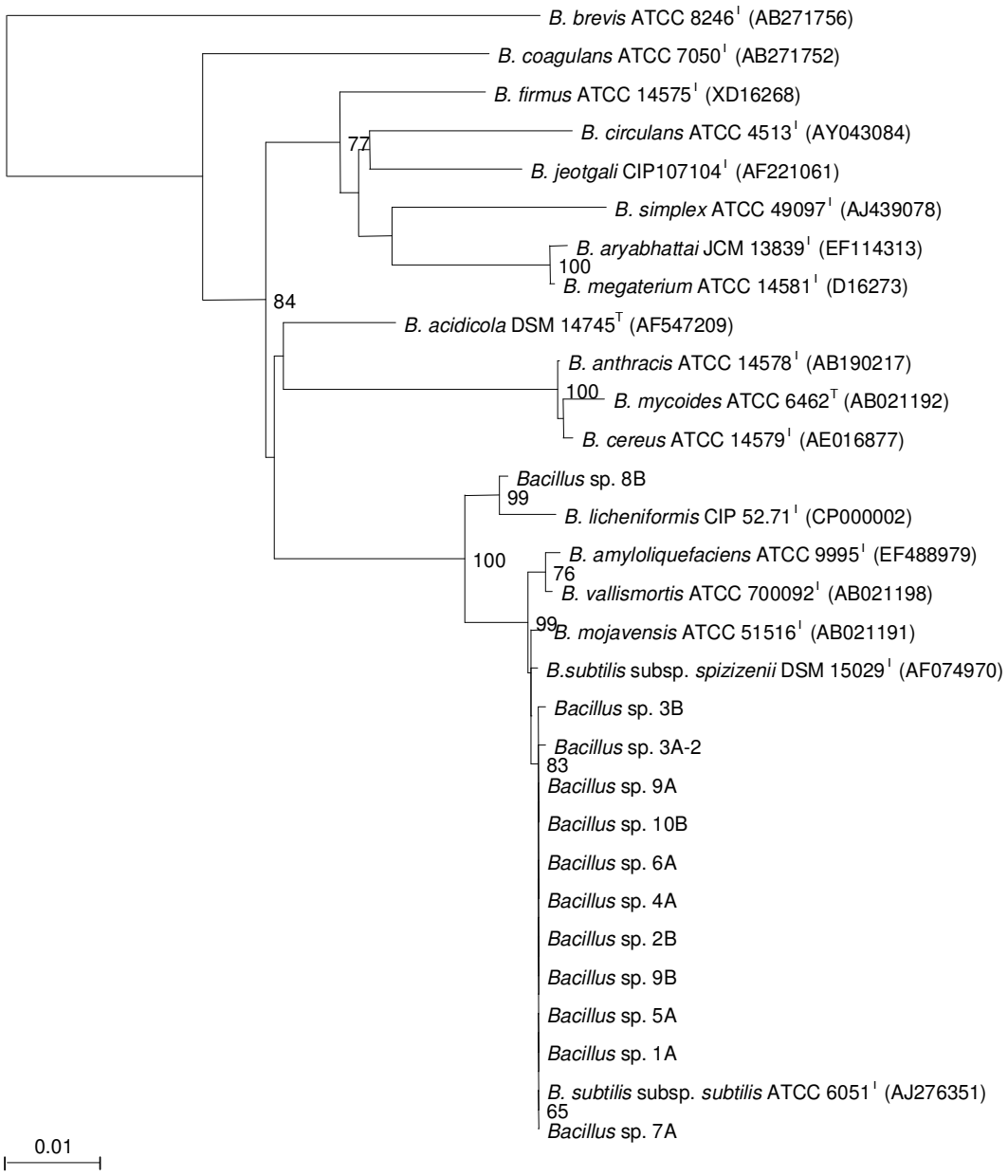

Figure 2. Phylogenic relationships of representative *Bacillus* strains obtained from 'iru' and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbor-joining method. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at the branch points. Bar, 0.01 substitutions per nucleotide position.

and 'ogiri' (Odunfa, 1981b; Beuchat., 1997; Kiuchi and Watanabe, 2004).

Taxonomic grouping of *Bacillus* species isolates

The relationship between the *Bacillus* species was determined by the rep-PCR fingerprinting method, a useful technique for determining inter- and intra-species relatedness (Versalovic et al., 1994; Gevers et al., 2001). The fingerprints obtained with primer $(GTG)_5$ are shown in Figure 1. The $(GTG)_5$ patterns resulted in the delineation at Pearson's correlation coefficient below 95% indicating that they were different genotypically and probably belong to the different species. Thus, on the basis of similarities in bands of the DNA dendogram, the isolates were divided into 12 groups (Table 3). Nick et al. (1999) and Rademaker et al. (2000) have compared rep-PCR genomic fingerprint analysis with DNA–DNA relatedness, they suggested that the two techniques yield

Table 3. Grouping of the *Bacillus* isolates from 'iru' on the basis of $(GTG)_5$ fingerprint.

Group	Isolate code	Group representative
I	3A-2, 9C	3A-2
II	1A, 2A, 3A	1A
III	7B, 8C, 9A	9A
IV	7A, 10A, 10C	7A
V	2B	2B
VI	6A	6A
VII	4A, 5A, 5B, 6B, 8A	4A, 5A
VIII	3B	3B
IX	4B	4B
X	1B, 9B	9B
XI	10B	10B
XII	8B, 8B-1	8B

results that are in close agreement. Heyrman et al. (2003) reported that rep-PCR fingerprinting can be used as a genomic screening method to differentiate at the species level and to select representatives for DNA–DNA reassociation experiments.

Figure 2 shows the phylogenic relationships of the *Bacillus* species from 'iru', with other type strains based on the 16S rRNA gene sequences. The 16S rRNA gene sequence similarities obtained showed that 3A-2, 1A, 9A, 7A, 2B, 6A, 4A, 5A, 3B, 4B, 9B and 10B (representative of Group I to XI, respectively) were most closely related to *B. subtilis subsp. subtilis* ATCC 6051^T with 99.5 to 100% similarity, while 8B (Group XII) was most closely related to *B. licheniformis* CIP 52.71^T (99.3%). Oguntoyinbo et al. (2004) reported variation in phenotypic and technological properties of 7 strains of *Bacillus* species involved in production of 'okpehe', with respect to production of extracellular enzymes, polyglutamate and bacteriocin. Further experiments are required to elucidate the differences in physiological properties of these isolates.

Conclusion

The 25 *Bacillus* species isolated from 10 commercial samples of 'iru' were grouped into 12 on basis of DNA fingerprinting. However, 16S rRNA gene sequencing has shown that they are closely related to *B. subtilis and B. licheniformis.*

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