International Journal of Food Microbiology 162 (2013) 95–104

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba

I. Ahaotu [a], A. Anyogu [c], O.H. Njoku [b], N.N. Odu [b], J.P. Sutherland [c], L.I.I. Ouoba [c,*]

[a] Department of Food Science, Federal University of Technology, Owerri, Nigeria
[b] Department of Microbiology, University of Port Harcourt, Rivers State, Nigeria
[c] Microbiology Research Unit, School of Human Sciences, Faculty of Life Sciences and Computing, London Metropolitan University, London, UK

ARTICLE INFO

Article history:
Received 17 August 2012
Received in revised form 19 December 2012
Accepted 2 January 2013
Available online 11 January 2013

Keywords:
African oil bean seeds
Ugba
Fermentation
Bacillus
Identification
Safety

ABSTRACT

Molecular identification of *Bacillus* spp. involved in the fermentation of African oil bean seeds for production of Ugba, as well as ability of the *Bacillus* spp. isolated to produce toxins, were investigated. Forty-nine bacteria were isolated from Ugba produced in different areas of South Eastern Nigeria and identified by phenotyping and sequencing of 16S rRNA, *gyrB* and *rpoB* genes. Genotypic diversities at interspecies and intraspecies level of the isolates were screened by PCR amplification of the 16S-23S rDNA intergenic transcribed spacer (ITS-PCR) and repetitive sequence-based PCR (rep-PCR). The ability of the bacteria to produce toxins was also investigated by detection of genes encoding production of haemolysin BL (*HblA*, *HblC*, *HblD*), non-haemolytic enterotoxin (*NheA*, *NheB*, *NheC*), cytotoxin K (*CytK*) and emetic toxin (*EM1*) using PCR with specific primers. Moreover, a *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination test kit (BCET-RPLA) was used to screen ability of the isolates to produce haemolysin in broth and during fermentation of African oil bean seeds.

The isolates were characterized as motile, rod-shaped, endospore forming, catalase positive, Gram-positive bacteria. They were identified as *Bacillus cereus sensu lato* (42), *Lysinibacillus xylanilyticus* (3), *Bacillus clausii* (1), *Bacillus licheniformis* (1), *Bacillus subtilis* (1), and *Bacillus safensis* (1). *B. cereus* was the predominant *Bacillus* species and was present in all samples studied. Using ITS-PCR, interspecies diversity was observed among isolates, with six clusters representing each of the pre-cited species. Rep-PCR was more discriminatory (eight clusters) and allowed further differentiation at intraspecies level for the *B. cereus* and *L. xylanilyticus* isolates with two genotypes for each species. Genes encoding production of non-haemolytic enterotoxin (*NheA*, *NheB*, *NheC*) and cytotoxin K (*CytK*) genes were detected in all *B. cereus* isolates, while Hbl genes (*HblA*, *HblC*, *HblD*) were detected in only one isolate. The emetic-specific gene fragment was not detected in any of the isolates studied. None of the toxin genes screened was detected in isolates belonging to other *Bacillus* species. Using RPLA, haemolysin production was detected in one isolate of *B. cereus*, which showed positive amplicons for Hbl genes, both during cultivation in broth and during fermentation of oil bean seeds.

1. Introduction

Ugba is produced from the alkaline fermentation of seeds of the African oil bean tree *Pentraclethra macrophylla* Benth. It is a large leguminous woody plant that belongs to the sub-family *Mimosoidae* (Keay, 1989). Ugba is produced and eaten mostly in the South-Eastern parts of Nigeria. The condiment is taken as a salad or added to soups and sauces as a flavouring agent. The fermented seed product 'Ugba' is traditionally prepared by boiling oil bean seeds overnight, slicing off the cotyledons, cooking these followed by washing them in water, soaking overnight and then fermenting the sliced cotyledons for a period of 3 to 5 days (Enujiugha, 2000).

Use of fermented vegetable proteins as seasonings and condiments is widespread in Africa and Asia (Steinkraus, 1996). There are several alkaline fermented legumes found in various parts of the world, e.g. Nigeria: Dawadawa, Ugba; Nepal: Kinema; Burkina Faso: Soumbala; Congo: Ntoba Mbodi; Thailand: Thuanao to mention a few (Odunfa, 1986; Barber et al., 1988; Sarkar et al., 1994; Isu and Ofuya, 2000; Louembe et al., 2003; Ouoba et al., 2004).

The production of Ugba, like other fermented foods in sub-Saharan Africa, is a traditional family art done in homes with local basic utensils. The fermentation process is spontaneous, usually with non-standard fermentation times and uncontrolled temperatures, leading to variable microbiological profiles, resulting in products of successive batches being different in quality attributes. This problem has been observed in most traditional fermented foods in

* Corresponding author at: Microbiology Research Unit, School of Human Sciences, Faculty of Life Sciences and Computing, London Metropolitan University, 166-220 Holloway Road, London N7 8DB, United Kingdom. Tel.: +44 20 7133 2155; fax: +44 20 7133 4149.
E-mail address: ouobairene@hotmail.com (L.I.I. Ouoba).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.01.001

Africa (Sanni, 1993). Occasionally, enabling factors in the microenvironment of the fermenting matrix may cause delay in fermentation and in some cases, fermentation may not be initiated (Sanni et al., 2002).

Use of starter cultures dominating the fermentation process and preventing growth of pathogenic and spoilage microorganisms is essential for controlled fermentation of such condiments in Africa. The potential of starter cultures for controlling and safe-guarding fermentation of traditional fermented foods has been emphasized (Holzapfel, 2002), including optimized production of condiments with a *Bacillus* starter (Beaumont, 2002). Starter culture selection is usually based on different metabolic behaviours during fermentation, such as proteolytic, lipolytic and amylolytic activities (Obeta, 1983; Odunfa, 1985; Ouoba et al., 2008a). *Bacillus* genera, species and strains that can bring about the expected and desirable biochemical changes in Ugba have not yet been accurately identified and a starter culture is not yet commercially available.

Microbiological studies of African fermented vegetable proteins established aerobic spore-forming bacteria, such as *Bacillus subtilis*, *Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus badius, Bacillus sphaericus*, *Bacillus cereus* and *Bacillus fusiformis* (Odunfa, 1981; Ouoba et al., 2004; Azokpota et al., 2006; Ouoba et al., 2008a; Parkouda et al., 2010) as the main frequently identified organisms and Ugba also belongs to this group of *Bacillus* fermented foods. Other microorganisms such as *Staphylococcus spp, Micrococcus spp, Lactobacillus plantarum, Streptococcus lactis, Proteus spp, Enterobacter spp.* and *Escherichia coli* implicated in the various fermentations have been reported but their role in the fermentation is yet to be determined (Odunfa, 1981; Barber et al., 1988; Sanni and Ogbonna, 1991; Oguntoyinbo et al., 2001).

The fermentation of Ugba is spontaneous by nature and may be carried out under unhygienic conditions; therefore the presence of pathogenic and spoilage strains of *Bacillus* cannot be totally ruled out in some fermentation batches. *Bacillus cereus* has been isolated from alkaline fermented foods such as Sonru, Iru, Afitin (Thorsen et al., 2010), Ogiri and Dawadawa (Okanlawon et al., 2010), Bikalga (Ouoba et al., 2008a) and Ugba (Mbajunwa et al., 1999). The presence of *Bacillus cereus* in these African fermented foods is a public health concern and should be investigated as it produces various toxins responsible of food poisoning accompanied with diarrhea and/or vomiting depending on the type of toxin produced. *Bacillus cereus* toxins include toxins such as heat labile non-haemolytic enterotoxin (Nhe), haemolysin (HBL), single protein cytotoxin CytK and heat stable emetic toxin (cereulide) (Granum et al., 1993; Agata et al., 2002; Michelet et al., 2006).

Methods that have been used for *Bacillus* identification and genotypic characterization include internal transcribed spacer polymerase chain reaction (ITS-PCR), random amplification polymorphic PCR (RAPD-PCR), repetitive sequence-based PCR (rep-PCR), pulsed field gel electrophoresis (PFGE) as well as sequencing of 16S rRNA, *gyrA*, *gyrB* and *rpoB* genes (Liu et al., 1997; Daffonchio et al., 1998; Da Silva et al., 1999; Yamada et al., 1999; Herman and Heyndrickx, 2000; Mendo et al., 2000; Parkouda et al., 2010; Thorsen et al., 2011a). Sequencing of 16S rRNA gene for identification of *Bacillus* shows limitations in differentiating closely related species and is used for basic classification for species delineation. However, it can be used in combination with, for example *rpoB* and *gyrB* genes sequencing which are more discriminatory, for improved identification and differentiation of closely related *Bacillus* species (De Clerck and De Vos, 2004; Wang et al., 2007).

There is a dearth of literature on genotypic identification of *Bacillus* spp. involved in fermentation of Ugba. To our knowledge, this is the first report on genotypic identification of *Bacillus* recovered from Ugba and consequential safety issues. The aim of this study was to identify *Bacillus* genera, species and strains isolated from Ugba by phenotyping and genotyping as well as to investigate possible toxin produced by the isolates to enable future selection of starter cultures for controlled fermentation.

2. Material and methods

2.1. Isolation of bacteria from Ugba

Bacteria were isolated from samples of Ugba prepared at various production sites in South Eastern Nigeria and from Ugba produced in the laboratory at London Metropolitan University (Table 2). For the latter procedure, African oil beans were purchased at Nkwo Ogwu market in Aboh Mbaise LGA in Imo state, Nigeria and brought to the UK. They were cleaned and pressure cooked for 3 h. The seeds were dehulled and the cotyledon sliced into thin long slices. The slices were boiled in water for 30 min and washed in clean cold water. The washed slices were then soaked in water for 12 h and drip dried before distributing 100 g aliquots into Stomacher bags. The seeds were allowed to ferment in an incubator at 37 °C, which is estimated to be close to the ambient temperature in the tropics. At 0 h, 24 h, 48 h, 72 h and 96 h of fermentation, samples were collected aseptically for microbial analysis along with the Ugba samples produced in Nigeria. The pH of all samples was also recorded. For microbial investigation samples were analyzed as follows: 10 g were homogenized for 1–2 min in 90 ml maximum recovery diluent (MRD; Oxoid CM0733, Basingstoke, UK). Ten-fold dilutions were prepared in MRD and 0.1 ml of suitable dilutions spread on nutrient agar (NA; Oxoid CM003). Plates were incubated aerobically at 37 °C for 48 h. Morphological characteristics of colonies on NA were examined and the number of colony forming units (CFU) recorded. Representative dominant colonies were isolated and purified by streaking several times on NA. Stock cultures were maintained in nutrient broth (NB; Oxoid CM001) containing 20% (v/v) glycerol (Sigma G8773, Gillingham, UK) and stored at −20 °C for further analyses.

2.2. Phenotypic characteristics

After purification, 49 bacteria were chosen from about 200 isolates based on their phenotypic characteristics after 24–48 h growth on NA (Oxoid CM003), including Gram and catalase reactions, cell morphology, cell motility and presence of endospores. Fermentation and assimilation of carbon compounds were determined using API 50 CHB kits (BioMerieux, Basingstoke, UK) according to the manufacturer's instruction and results were analyzed using the API Software (APIWeb, Biomerieux).

2.3. Genotypic identification

2.3.1. Extraction of DNA

Each isolate was streaked on Tryptone Soya Agar (TSA; Oxoid CM0131) and incubated at 37 °C for 48 h. Chromosomal DNA of each isolate was extracted using InstaGene™ Matrix (Bio-Rad 732–6030, Hemel Hempstead, UK) according to manufacturer's instructions and stored at −20 °C until required.

2.3.2. Differentiation of isolates at species and intraspecies level

The isolates were differentiated to species level as previously described (Ouoba et al., 2008a) by amplifying the 16S-23S rDNA intergenic transcribed spacer (ITS-PCR) using primers S-D-Bact-1494-a-S-20-F and L-D-Bact-0035-a-A-15-R (10 pmol/μl, Table 1) under the following conditions: 94 °C for 1 min followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The PCR ended with a final extension at 72 °C for 7 min and the amplified product cooled at 4 °C. Intraspecies diversity among the isolates was further investigated by rep-PCR as previously described (Ouoba et al., 2008a) using primer GTG5 (5 pmol/μl, Table 1) under the following conditions: 94 °C for 4 min followed by 30 cycles at 94 °C for 30 s, 45 °C for 1 min and 65 °C for 8 min. The PCR ended with a final extension at 65 °C for 16 min and the amplified product cooled to 4 °C.