Advances in Environmental Biology, 5(9): 2960-2966, 2011
ISSN 1995-0756



**ORIGINAL ARTICLE**

# Numeration and Identification of thermotolerant endospore-forming *Bacillus* from two fermented condiments *Bikalga* and *Soumbala*

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Savadogo Aly, Ilboudo A. Jules, Gnankiné Olivier, Traoré Alfred S**.** Numeration and Identification of thermotolerant endospore-forming *Bacillus* from two fermented condiments *Bikalga* and *Soumbala*

**ABSTRACT**

The thermotolerant *Bacillus* associated with *Bikalga* and *Soumbala* process were screened numbered and identified using standards and PCR methods. Thermotolerant bacteria in *Soumbala* ranged from 1.35 x $10^9$ to 3 x $10^9$ cfu/g, 1.24 x $10^9$ to 2.78 x $10^9$ cfu/g, 0.54 x $10^9$ to 2.27 x $10^9$ cfu/g, 3.5 x $10^7$ to 1.54 x $10^9$ cfu/g respectively for 90°C, 95°C, 100°C and 105°C . Thermotolerant bacteria in *Bikalga* ranged from 1.9x$10^7$ to 12.8x $10^7$ cfu/g, 2x$10^6$ to 11.2 x $10^7$ cfu/g, 0 to 9.6 x $10^7$ cfu/g, 0 to 7.6 x $10^7$ cfu/g respectively for 90°C, 95°C, 100°C and 105°C. Sixteen strains were selected and identified as belonging to *Bacillus* genera. All these selected strains are Gram positive, catalase positive and endospore forming, among these selected strains *Bacillus coagulans*, *Bacillus cereus*, *Bacillus pumilis*, *Bacillus firmus*, *Bacillus subtilis and Bacillus licheniformis* were identified. *Bacillus* is the predominant genera in these two fermented condiments called *Soumbala* and *Bikalga*. *Bacillus subtilis*, *Bacillus cereus, Bacillus pumilus* were identified in *Soumbala* and *Bacillus licheniformis, Bacillus cereus, Bacillus pumilus, Bacillus coagulans , Bacillus firmus* were identified in *Bikalga.* These *Bacillus* species exhibited pronounced activities of protease and amylase in fermented condiment. *Bacillus* species have been reported by several authors as important micro-organisms in fermented condiments foods. The isolates were assayed for protease and amylase production and activity.

***Key words:*** *Bikalga, Soumbala, Bacillus,* Thermoterant, Numeration, identification

## Introduction

*Bikalga* and *Soumbala* are condiment products of traditional uncontrolled alkaline fermentation of *Hibiscus sabdariffa and Parkia biglobosa* seeds respectively. These food additives are used and produced as major condiments in many African countries including Burkina Faso, Cameroon, Mali, Niger, Senegal, Sierra Leone and Sudan among others. *Bikalga* and *Soumbala* are produced by women and constitute economical resource. *Soumbala* is known under different appellations depending of the country: *dawadawa or iru* in Nigeria and North of Ghana [26,37,11], *nététou* in Sénégal [23], *afitin* in Bénin [4], *kinda* (Sierra Leone), *natto* in Japan and *kinema* in Nepal [5,38]. *Bikalga* is also called *Dawadawa botso* in Niger [34], *Datou* in Mali [34], *Furundu* in Sudan [40], *Mbuja* in Cameroon [22]. They are excellent sources of proteins with essential amino acids also containing lipids, carbohydrates, essential fatty acids and vitamins[6,33,40]. Many families in west Africa often used *Soumbala* and

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*Bikalga* as low-cost meat substitute. These condiments improve nutritional values of foods as well as sensory properties as taste enhancer; contain antioxidant and neutraceuticals that provide health.

Several works were dedicated to the characterization of the microflora of these fermented products [11,4,32,17,21,29]. These studies demonstrated the prevalence of *Bacillus* species (*B. subtilis*, *B. coagulans*, *B. amyloliquefaciens*, *B. pumilus*, *B. cereus*, *B. thuringiensis*, *B. brevis* and *B. licheniformis*) in the fermentation process and their role in the bioconversion of the products. Ouoba *et al*. [32] in their recent studies sowhed that *Bacillus subtilis*, *Bacillus licheniformis*, *B. pumilus*, *B. cereus*, *B. badius*, *B.sphaericus* and *B. fusiformis are* microorganisms present and involved in *Bikalga* fermentation. Parkouda *et al.* [34] were showed that these condiments contained several *Bacillus* strains which play a key role during fermentation for the manufacture of the product. Micro-organims involves or responsible for the fermentation of *Soumbala* have been identified as *Bacillus* spp. with *B. subtilis* as the predominant species [26,2,25,12,24].

The initial boiling of *Parkii biglobosa* and *Hibiscus sabdariffa* seeds during their traditional preparation process of these two Condiments may be way of thermotorant microorganisms selection and destroyed non-*Bacillus* species; this could explain the dominance of *Bacillus* species in *Soumbala* and *Bikalga*. However there is no information or few data on numeration of the thermotorant flora of these two condiments well consumed in Burkina Faso. This work deals with numeration and identification of thermotorant bacteria from *Soumbala* and *Bikalga*.

## Materials and methods

*Collection of Samples:*

*Bikalga* and *Soumbala* samples were bought from various small markets (Table1) of Ouagadougou and Gaoua. Samples were transported at the microbiology laboratory immediately after collection and analysed or stored under refrigeration until analysis.

*Microbial Analysis:*

10 g of each sample was placed in a sterile stomacher bag containing 90 ml of peptone saline water and subsequent serial dilution up to $10^{-10}$ of each sample was made. Before analysis each sample (10 g plus 90ml peptone saline water) was heated at 90°C, 100°C, 105°C for 15 minutes. 0.2 ml of each appropriate dilution was inoculated triplicate in Plate Count Agar and incubated at 37°C for 24 H for viable colonies count. After incubation developed colonies on agar were counted for each sample.

*Micro-organisms Primary Characterization:*

To confirm that the isolated colonies are belonging to *Bacillus* species different tests were made. Sixten bacteria colonies from *Soumbala* and *Bikalga* were selected (5 from *Soumbala* and 11 from *Bikalga*), isolated and characterized according to methods described by Harrigan and McCance [13], ICSMF [15]; Collins and Lyne [10]. After colony counting different colonies were picked at random from petri dish and identified by cell morphology and motility, gram reaction, glucose metabolism [14], lecithinase, VP (Voges-Proskauer test), urease, catalase, starch hydrolysis, casein hydrolysis, sporulation, NaCl.

*Selected Strains Characterization by PCR using Bacillus genus Specific Primer:*

*DNA Extraction and Preparation*:

5ml of an overnight culture were used, cells were harvested by centrifugation and washed twice with Nacl 0.9%. Isolation of DNA was conducted with *PROMEGA* kit and protocol technics (Promega Corporation, Madison, WI 53711-5399 USA).

*PCR Reaction and Electrophoresis*:

Primers (B-K1/R: 5'-TCACCAAGGCRACGATGCG-3' and B-K1/F: 5'-CGTATTCACCGCGGCATG-3') were used for the identification of *Bacillus* strains [39]. PCR mixture consisted of 1.5 µl of each primer (20 µM), 12.5 µl (1.2 µM) of Master Mix (Fermentas GMBH, St-Leon, Rot, Germany), 8 µl of $H_2O$, 1.5 µl of DNA (10-30 ng.$\mu l^{-1}$) in a final volume of 25 µl. Thermal cycling was carried out using a Eppendorf AG (Hamburg, German) Mastercycler gradient as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1min, primer annealing at 43°C for 30 s primer extension at 72°C for 45 s and final extension at 72°C for 10min. The expected fragment size is 1114bp. 10µl of the amplified products of PCR were analysed by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide (0.5 mg $ml^{-1}$). The gels were visualised with an Ultraviolet Illuminator and Digitally recorder (GelDOC Bio-Rad, Hercule, USA).

*Dermination of Proteolytic Activity:*

Proteolytic activity of the isolates was determined using skim milk agar. The culture supernatant were spotted on the surface of the skim milk agar plates and incubated at 37°C for 24h. The development of a clear zone was considered as proteolytic activity [8].

**Table 1:** Sampling of *Bikalga* and *Soumbala*

| Samples Codes | samples | Number of sample | Place of sampling |
|---|---|---|---|
| S1, S3, S4, S6, S9, S10 | *Soumbala* | 6 | Zogona market |
| B1,B2, B5, B7, B10 | *Bikalga* | 5 | (Ouagadougou) |
| S2 | *Soumbala* | 1 | Somgandé market (Ouagadougou) |
| S5 | *Soumbala* | 1 | Goua market (Gaoua) |
| S7 | *Soumbala* | 1 | Nabigyaar market |
| B9 | *Bikalga* | 1 | (Ouagadougou) |
| S8 | *Soumbala* | 1 | Zone I market |
| B6 | *Bikalga* | 1 | (Ouagadougou) |
| B3, B4 | *Bikalga* | 1 | Karpala Market(Ouagadougou) |
| B8 | *Bikalga* | 1 | Goughin (Ouagadougou) |
| | | | S=*Soumbala*, B= *Bikalga* |

*Amylolytic Activity:*

For determination of amylolytic activity starch agar was used and incubated at 37°C for 48h. Enzymatic activity was indicated as clearing zones on the plates. For observation of amylolytic activity the agar plates were flooded with iodine solution.

## Results and Discussion

*Numeration:*

The count of thermotolerant bacteria in *Soumbala* (Table 2) at 90°C, 95°C, 100°C and 105°C ranged from 1.35 x $10^9$ to 3 x $10^9$ cfu/g, 1.24 x $10^9$ to 2.78 x $10^9$ cfu/g, 0.54 x $10^9$ to 2.27 x $10^9$ cfu/g, 3.5 x $10^7$ to 1.54 x $10^9$ cfu/g respectively. Thermotolerant bacteria in *Bikalga* (Table 3) at 90°C, 95°C, 100°C and 105°C ranged from 1.9x$10^7$ to 12.8x $10^7$ cfu/g, 2x$10^6$ to 11.2 x $10^7$ cfu/g, 0 to 9.6 x $10^7$ cfu/g, 0 to 7.6 x $10^7$ cfu/g. These results indicated that *Soumaba* contained more themotolerant bacteria than *Bikalaga*. In all samples samples of *Bikalga* and *Soumabala* we numbered thermotolerant bacteria at 90°C, 95°C, 100°C and 105°C, except in of *Bikalga* samples B2 at 10°5C and B3 at 100°C and 105°C we didn't numbered no themotolerant . This analysis of heat treatment microbial flora in *Bikalga* and *Soumbala* revealed the occurring of both *Bacillus* genera. Colonies morphology on nutrient agar clearly indicates the presence of more than one type of isolate, confirming thus the diversity of flora.

*-Primary Characterization, Proteolytic and Amylolytic Activities:*

Five (5) representative strains from *Soumbala* and eleven (11) representative strains from *Bikalga* were selected for morphological and biochemical characterization (Table 4). All selected strains are Gram-positive, endospore-forming catalase positive; these selected strains are identified according to Biochemical, morphology and PCR characteristics (Table 4): five (5) are *Bacillus coagulans*, four (4) are *Bacillus cereus*, three (3) are *Bacillus pumilis*, two (2) are *Bacillus firmus*, one (1) is *Bacillus subtilis* and one (1) is *Bacillus licheniformis*. *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pumilus* were identified in *Soumbala* and *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus coagulans*, *Bacillus firmus* were identified in *Bikalga* (Table 4). Further examination (Table 4) showed that the selected strains hydrolysed casein (15 strains) and starch (6 strains), grew in medium with 5 % to 8% NaCl (10 strains for 5% NaCl, 6 strains for 6% NaCl, 5 strains for 7% NaCl and 3 strains for 8% NaCl). The heating has destroyed the non-*Bacillus* species and others microorganisms in volved in *Soumbala* and *Bikalga* process. Ouoba *et al.* [33] reported extra and intracellular protease enzymes pronounced activity in *Bacillus* species. *Bacillus* counts in soybean *dawadawa* by Ogbadu and Okagbue [25], Opai-Tetteh [30] and Omafuvbe *et al.* (2000) were estimated in order of $10^{10}$ to $10^{11}$ cfu/g.

*PCR Characterization:*

The amplification with PCR using *Bacillus* genus primers gave expected fragments 1114 bp for the sixteen selected strains (Figure 1). These results confirm that the selected strains are effectively belonging to the *Bacillus* genus.

*Discussion:*

The count of bacteria was dominated by Gram-positive, catalase-positive, rods, endospore-forming all these characteristics are those of *Bacillus* group , this fact is due to heating during the numeration at 90°C ,95°C, 100°C and 105°C for 15 minutes. The initial boiling of *Parkii biglobosa* and *Hibiscus sabdariffa* seeds during their traditional preparation process of these two condiments and the heating during the numeration of microorganisms in our samples may be way of thermotorant microorganism selection and destroyed non-*Bacillus* species; this could explain the dominance of *Bacillus* species in *Soumbala* and *Bikalga*. Also The pH of these two condiments are alkaline this fact can explain why we get otptimum spore heat resistance and high number of *Bacillus* counted.

Among Sixteen (16) selected strains 15 have proteolytic activity this result indicate that *Bikalga* and *Soumbala* are rich in proteolytic *Bacillus* spp.

**Table 2:** Microbial analysis of *Soumbala* after treatment at different temperature.

| *Soumbala* samples | 90°C | 95°C | 100°C | 105°C |
|---|---|---|---|---|
| | | CFU $g^{-1}$ | | |
| S1 | 2.76 x$10^9$ | 2 x$10^9$ | 1.51 x$10^9$ | 3.5 x$10^7$ |
| S2 | 2.81x $10^9$ | 1.8 x$10^9$ | 1.56 x$10^9$ | 1.10 x$10^9$ |
| S3 | 2.96 x$10^9$ | 2.56 x$10^9$ | 2.02 x$10^9$ | 1.54 x$10^9$ |
| S4 | 2.89x $10^9$ | 2.69x $10^9$ | 2.27 x$10^9$ | 5.8x $10^8$ |
| S5 | 3 x$10^9$ | 2.78 x$10^9$ | 2.19x $10^9$ | 1.21x$10^9$ |
| S6 | 2.45x$10^9$ | 2.37x $10^9$ | 1.98x $10^9$ | 6.7x $10^8$ |
| S7 | 1.84x$10^9$ | 1.24x $10^9$ | 6.2 x$10^8$ | 3.8 x$10^7$ |
| S8 | 2.84 x$10^9$ | 1.65 x$10^9$ | 7.1 x$10^8$ | 5x$10^8$ |
| S9 | 1.4 x$10^9$ | 1.28x $10^9$ | 8.9x $10^8$ | 6.2 x$10^8$ |
| S10 | 1.35 x$10^9$ | 1.3x $10^9$ | 5.4x $10^8$ | 1.12x$10^8$ |

Values are weighted means of three replicates

**Table 3:** Microbial analysis of *Bikalga* after treatment at different temperature.

| *Bikalga* samples | 90°C | 95°C | 100°C | 105°C |
|---|---|---|---|---|
| | | CFU $g^{-1}$ | | |
| B1 | 2.3x$10^7$ | 2.2 x$10^7$ | 2.1x$10^7$ | 1x$10^7$ |
| B2 | 3.6 x$10^7$ | 4.5 x$10^7$ | 3.2x $10^7$ | 0 |
| B3 | 1.9 x$10^7$ | 2. $10^6$ | 0 | 0 |
| B4 | 4.5x $10^7$ | 7.1x $10^7$ | 5.6x$10^7$ | 4.4x $10^7$ |
| B5 | 5 x$10^7$ | 6x $10^7$ | 5.5x $10^7$ | 7 x$10^6$ |
| B6 | 3.5x $10^7$ | 2.2x $10^7$ | 1.3x $10^7$ | 1.2x $10^7$ |
| B7 | 6 x$10^7$ | 5.3x $10^7$ | 4.2 x$10^7$ | 2.7 x$10^7$ |
| B8 | 10x$10^7$ | 6.9x $10^7$ | 6.8x$10^7$ | 2x$10^7$ |
| B9 | 12.5x$10^7$ | 11.2x$10^7$ | 3.2x$10^7$ | 2.9x$10^7$ |
| B10 | 12.8x$10^7$ | 10x$10^7$ | 9.6 x$10^7$ | 7.6 x$10^7$ |

Values are weighted means of three replicates

**Table 4:** Biochemical and morphology characters of isolates of *Soumbala* and *Bikalga*.

| | Isolated at 90°C | | | | Isolated at 95°C | | | Isolated at 100°C | | | | Isolated at 105°C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Characters | S1 | B1 | B2 | B3 | S2 | S3 | B4 | B5 | S4 | B6 | B7 | B8 | S5 | B9 | B10 | B11 |
| Gram | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 11 |
| Catalase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 |
| Sporulation | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 |
| Voges Proskauer | + | - | + | + | - | - | + | + | + | - | + | - | + | + | + | - |
| Lecihinase | - | - | + | - | - | + | - | - | - | - | - | - | + | - | + | - |
| Starch hydrolysis | - | - | - | - | +13mm | +13mm | +16mm | +16mm | - | +17mm | +15mm | +16mm | - | - | - | - |
| Casein hydrolysis | +14mm | +10mm | +18mm | +14mm | +10mm | +8mm | +10mm | +10mm | +9mm | -0 mm | +12mm | +4mm | +11mm | +14mm | +6mm | +6mm |
| Urease | - | - | - | + | - | + | - | - | - | + | - | - | + | - | - | - |
| NaCl (5%) | + | - | - | + | + | + | + | - | + | + | - | + | - | - | + | 6 |
| NaCl (6%) | + | - | - | + | + | - | + | - | - | + | - | - | - | - | + | - |
| NaCl (7%) | + | - | - | + | + | - | + | - | - | - | - | - | - | - | + | - |
| NaCl (8%) | - | - | - | + | - | - | + | - | - | - | - | - | - | - | + | - |
| Presumptive Identification | *B. pumilus* | *B. coagulans* | *B. cereus* | *B. pumilus* | *B. subtilis* | *B. cereus* | *B. licheniformis* | *B. coagulans* | *B. pumilus* | *B. firmus* | *B. coagulans* | *B. firmus* | *B. cereus* | *B. coagulans* | *B. cereus* | *B. coagulans* |

+= positive reaction ; -= negative reaction ; B= strains from *Bikalga* (B1, B2,B3, B4, B5, B6, B7, B8, B9, B10); S= strains from *Soumbala*( S1, S2, S3, S4, S5)

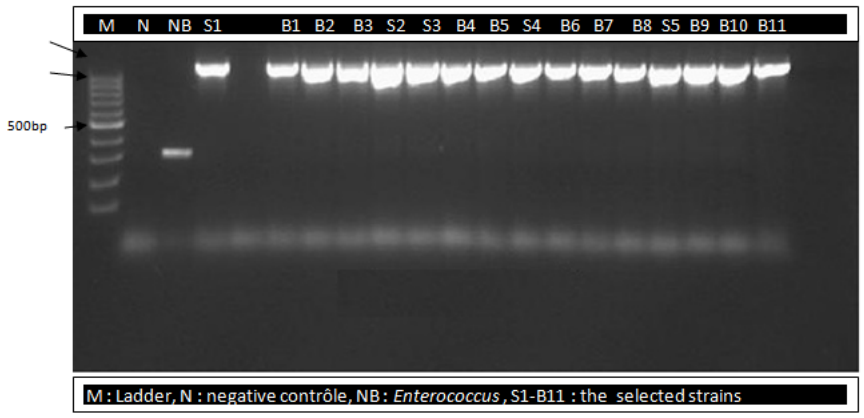


**Fig. 1:** Electrophoresis gel with PCR using primers BK1-R and BK1-F products.

*Bacillus* spp. was found to be dominant in *iru* and *sonru* [3] in *dawadawa* [25] in *Soumbala* [12,5,33,31] in *Meju* and others fermented soybean foods [19,9,20,18]. Lipolysis and proteolysis are important processus observed in fermented condiments [1,33]. *Bacillus subtilis* was encountered and used as starter culture for production of locust bean daddawa [16,27].

*B. subtilis* was encountered and used as starter culture for production of locust bean daddawa [16,27].

Proteins degradation in fermented condiment contributes to the development of the texture, flour and organoleptic quality. Lipolysis and proteolysis are important processus observed in fermented condiments [1,39,33]. Proteolytic activity of *Bacillus* can lead to the liberation of bioactive compounds which could give somes interesting role and characteristics of fermented condiments. The production of several proteolytic enzymes enhance the growth of *Bacillus* species in fermented food [21]. The hydrolysis of protein during *Bikalga* and *Soumbala* fermentation leads to the alkalinity due to the protease and deaminase enzymes produced by the *Bacillus* group. Amylase activity has been reported to be the second most important activity during condiment production. Strain with protease activity will be relevant for flavor development and release of amino acids during condiment process. Strains with good protease and amylase will be more relevant as starter culture.

The *Bacillus spp* play important role in *Maari* fermentation [35] such as development of aroma and flavor. *Bacillus subtilis* was also present in others similar alkaline fermented condiments such as *afitin* [4] *dawadawa* [11] iru [26], *Soumbala* [31] *kinema* [36] *mbuja* [22]. *Bacillus subtilis* and *Bacillus licheniformis* members of *Bacillus* group have same ability to degrade locust bean proteins. Proteins degradation is likely to produce essential amino acids and bioactive peptides useful for human nutrition.

Fermented soybean foods have several functional properties like antioxidant, antimutagenesis, immunomodulatory, antithrombosis and fibrinolytic [7,9].

*Conclusion:*

This work indicates that *Bacillus* group are endospore-forming thermotolerant bacteria involved in Soumbala and Bikalaga fermentation and contribute to their sensor and nutritional characteristics. Enumeration result shows that the thermoteroleant bacteria are important and dominant in these two condiments. These result can be explain by the fact that *Bikalga* and *Soumbala* are condiments product of traditional uncontrolled alkaline fermentation of *Hibiscus sabdariffa and Parkii biglobosa* seeds respectively after long time cooking process.

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