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# Some technological properties of selected strains of *Bacillus* spp. associated with kantong production in Ghana

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**Abstract**

This study investigated some technological properties of *Bacillus* spp. involved in fermenting *Ceiba pentandra* seeds into kantong a condiment in northern Ghana with a view to selecting some for starter culture development to aid commercial production of kantong. *Bacillus amyloliquefaciens, B. safensis, B. altitudinis, B. thuringiensis, B. pumilus, B. megaterium, B. cereus, B. circulans, B. coagulans, B. firmus, B. subtilis*, and *B. licheniformis* which predominated two hundred and five *Bacillus* strains isolated from eleven stages of kantong production were assessed for some technological properties such as proliferation temperature and pH; substrate utilization preferences and inhibitory activity against selected pathogenic and spoilage organisms. Test strains proliferated at temperatures between 10°C and 55°C and pH of 2 to 9. Substrate utilization preferences were Nutrient Agar with 5-9% Sodium Chloride [NA/NaCl], and ordinary Nutrient Broth [NB], Nutrient Agar [NA], Potato Dextrose Agar [PDA], Tryptone Soya Agar[TSA] and MacConkey Agar [MCA]. All strains exhibited inhibitory activity against one or more pathogenic and spoilage organisms, *Salmonella typhimurium* being the most susceptible. Many strains qualified as potential candidates for selection and development as starter cultures to be used in the commercial production of kantong of consistent and acceptable organoleptic quality.



## Introduction

Fermented foods are agricultural products which have been converted by enzymatic activities of microorganisms into desirable food products whose properties are considered more attractive than those of the original raw materials (Kpikpi, 2006). All around the world, fermented foods and beverages are part of the human diet. Traditional fermented products are of major importance in the diet of Africans. Ghana has a rich stock of indigenous fermented foods including fermented fish, cowhide, and crabs. Fermented cereals and tubers such as maize and cassava are also fermented for use in local staples like kenkey, koko, and banku, etc. Fermented tree seeds give rise to condiments such as dawadawa and kantong.

Fermentation in food processing typically is the conversion of carbohydrates to alcohol and carbon dioxide using yeast, bacteria, or a combination thereof, under anaerobic conditions. It offers several advantages such as improved flavour, prolonged shelf-life, better food safety, and increased bioavailability of proteins and micronutrients.

Various studies have been conducted to evaluate the microorganisms involved during the fermentation of cassava (Ampe *et al*., 1999; Ben and Ampe, 2000; Coulin *et al*., 2006). Predominant organisms isolated at the beginning of fermentation of cassava were *B. subtilis, B. mycoides, B. pumilus, B. cereus, B. amyloliquefaciens* and *B. licheniformis* (Gbenga *et al*., 2009). The breakdown of cassava texture occurs through the hydrolysis of cassava tuber cellulose by cellulases produced by these organisms, bringing about modification of texture of the cassava (Amoa-Awua and Jakobsen, 1996). Alkaline fermentations by *Bacillus* spp. such as *B. subtilis* (dominant species), *B. licheniformis* and *B. pumilus* cause the hydrolysis of protein to amino acids and peptides, releasing ammonia, which increases the alkalinity of the final product (Enujiugha *et al.*, 2008; Chelule *et al.*, 2010).

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According to Adegbehingbe (2013), dehulled and ground white cultivars of cooked, pressure-cooked and uncooked Lima bean seeds were fermented in calabashes for nine days. Microorganisms isolated from the samples included *B. subtilis, B. megaterium, B. polymyxa, B. pumilus, B. licheniformis, Lactobacillus plantarum, Aspergillus fumigatus* and *Saccharomyces cerevisiae*. Six predominant bacterial species isolated during fermentation of ogiri, a seed condiment from three types of melon seeds (*Cucumeropsis manii* (Naud); *Citrullus lanatus* (L) and *Colocynthis vulgaris* (Schrad) were found to belong to the genera *Bacillus, Micrococcus, Leuconostoc, Streptococcus, Pediococcus* and *Lactobacillus* (David, 2010). During production of traditional fermented condiments like dawadawa, iru and ogiri, the microorganisms use the nutritional components of the seeds, converting them into products that contribute to the chemical composition and taste of the condiment. The spore-forming species *B. subtilis* and *B. licheniformis* were identified as the main bacteria present (Achi, 2005).

Most of the traditional foods in West Africa are fermented before consumption (Odunfa, 1985). The production of these indigenous foods is plagued with problems such as inconsistent quality, hygienic risks and short shelf life because their preparations generally depend on chance inoculation from the environment and the use of starter cultures is not common (Onyekwere *et al*., 1989). In spontaneous fermentation there is a high risk of contamination. If fermentation is not properly conducted, spoilage may appear which causes bad odour and bad taste (butyric acid, hydrogen sulphide, aromatic amines). Also, there is a danger of contamination by pathogenic bacteria such as *Shigella, Salmonella, Campylobacter, Pseudomonas, Listeria, Vibrio vulnificus, Clostridium botulinum, Escherichia coli,* and *Staphylococcus aureus*. Risk of intoxication is another pitfall of spontaneous fermentation. There were reported cases of dangers associated with the consumption of fermented food. In Alaska, fish, seafood and birds were traditionally fermented in grass-lined hole. In the 1980's the fermentation began to be carried out in plastic containers. This resulted in the development of botulinum bacteria which thrived under anaerobic conditions and caused several botulism cases (Fellows, 2000; Mirbach and El Ali, 2005).

To improve the microbiological safety of West African fermented foods, the use of bacteriocin-producing lactic acid bacteria in fermentation was suggested (Olukoya *et al*., 1993). According to Jespersen (2003), due to the lack of know-how, appropriate technology and infrastructure, fermented foods are still primarily produced by spontaneous fermentations without the use of starter cultures and controlled conditions.

In Nigeria, Burkina Faso and the northern parts of Ghana, seeds of the African locust bean *(Parkia biglobosa)* and Silk Cotton *(C. pentandra)* have been reported to be fermented and used as condiments, namely iru from Nigeria (Odunfa,1988), soumbala from Burkina Faso (Ouoba *et al*., 2003) and kantong from northern Ghana (Kpikpi, 2006). Kantong is a fermented seed-cake (condiment) primarily prepared from the seeds of *C. pentandra* (silk cotton) and is used predominantly by the Dagombas of northern Ghana. The seeds are pounded into flour and sifted, and then cassava flour and water are added to make a thick paste. It is then fermented for 48 hrs, after which it is dried, pounded and moulded into a cake and is ready for consumption (Kpikpi, 2006).

In contrast to the many reported studies on fermented products from *P. biglobosa* (Odunfa 1981, 1985; Odunfa and Adewuyi, 1985; Odunfa and Oyewole, 1986; Ouoba *et al*., 2004, 2005 and 2007), only one study has been reported on the microbiology of kantong production from *C. pentandra* in northern Ghana (Kpikpi, 2006). This study found out that microorganisms fermenting *C. pentandra* seeds into kantong included lactic acid bacteria and *Bacillus* spp. Metabolic activities of *Bacillus* spp. are generally believed to release ammonia which imparts a pungent odour to the final product, the unique flavour and aroma, desired by indigenous patrons. However, no previous study has been reported that investigated this and other desirable technological properties of *Bacillus* spp. found to be associated with kantong production.

To make kantong appealing and acceptable to non-indigenes as well, there is the need to regulate its odour and consistency through controlled fermentation using starter cultures developed from the associated *Bacillus* spp. Starter culture development is dependent upon knowledge of the technological properties of a candidate organism. This study sought to investigate some technological properties of *Bacillus* spp. associated with kantong production such as proliferation temperature and pH, substrate utilization preferences, and inhibition of pathogenic bacteria, to enable selection of suitable candidates for development as starter cultures for commercial production of kantong with acceptable and consistent organoleptic quality.

## Materials and Methods

*Source of microorganisms*

The *Bacillus* strains investigated were isolated from different stages of kantong production from two areas of the Northern region of Ghana designated as Nyankpala North and Nyankpala South and identified as described by Kpikpi *et al.* (2014). Predominant among these were *B. amyloliquefaciens, B. safensis, B. altitudinis, B. thuringiensis, B. pumilus, B. megaterium, B. cereus, B. circulans, B. coagulans, B. firmus, B. subtilis,* and *B. licheniformis*. Five of the indicator organisms used in the inhibitory test, *E. coli* (i9), *Klebsiella pneumoniae* (g8i), *Acinetobacter baumannii* (t3), *Enterococcus cloacae* (h7), and *Enterococcus faecium* (e5i) were also isolated from kantong and identified as described by Kpikpi *et al.* (2014) while three others,*Pseudomonas aeruginosa* (DSM 939), *Listeria monocytogenes* and *E. coli* (DSM 682) were reference strains of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany, got from the Department of Food Science of the University of Copenhagen, Denmark. *Sal. typhimurium* was a reference strain got from the University Hospital, Copenhagen while *Proteus vulgaris, Sh. flexneri* and *Staphylococcus aureus* were sourced from the University for Development Studies (UDS) Microbiology Laboratory, Navrongo. While the first five indicator organisms isolated from kantong were used because of their probable presence as spoilage organisms, all others were selected based on their reported implication in food-borne illness.

Three strains each of the predominant species---*B. amyloliquefaciens* (O1, O2, O4); *B. safensis* (T9, S5, J9); *B. altitudinis* (I1, R2, L10) and *B. subtilis* (K2, N6, Q7)---were investigated for their inhibitory activities against twelve indicator microorganisms, namely *E. coli* (DSM 682), *E. coli* (i9), *K. pneumoniae* (g8i), *Ac. baumannii* (t3), *Ent. cloaclae* (h7), *Sal. typhimurium, P. aeruginosa* (DSM 939), *Pr. vulgaris, Ent. faecium* (e5i), *Sh. flexneri, L. monocytogenes* and *Staph. aureus.*

*Preparation of inocula of* Bacillus *spp. and pathogenic microorganisms*

From NA (OXOID, Basingstoke, Hampshire, England) plates incubated at 37°C for 24hrs, the various strains of *Bacillus* spp. and the indicator microorganisms were sub-cultured in 10ml of NB (OXOID, Basingstoke, Hampshire, England) and incubated at 37°C for 24hrs, followed by streaking onto NA (OXOID) and incubated under same conditions. Pure cultures obtained were grown on agar slants in Eppendorf tubes and stored at -20°C until use. The cell-free supernatant fluids were prepared according to Bonadè *et al*. (2001). The number of cells /ml was estimated by microscopy using a counting chamber (Neubauer, Wertheim, Germany) and dillutions were made in sterile saline (0.85%, w/v NaCl) to obtain a rate of inoculation of 105 – 106 cells /ml.

The antimicrobial-producing strains being investigated were grown in 50 ml of Brain Heart Infusion Broth [BHIB] (OXOID, Basingstoke, Hampshire, England) at 37°C with agitation at 120 rpm for 18hrs. Supernatant fluids were collected by centrifuging the cultures at 5000 x g for 10min at 4°C and the pellets suspended in 5ml saline solution containing 8.5 g/l NaCl and 1.5 g/l Bactopeptone (DIFCO, Detroit MI, USA). The pH of the supernatants was adjusted to 7 using Sodium Hydroxide (NaOH) and concentrated Hydrochloric Acid (HCl) as appropriate, and treated with 5 mg / ml catalase to eliminate hydrogen peroxide and filter-sterilized using a 0.2 µl pore size filter (FP 030/3 Schleicher and Schuell GmbH, Dassel, Germany) (Bonadè *et al*., 2001).

*Investigation of technological properties*

NA(OXOID) plates surfaced-streaked with the isolates were incubated at temperatures of 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 45°C, 50°C, 55°C and 60°C for 24hrs and observed for growth. The isolates were also inoculated on MCA (MERCK), PDA(OXOID), TSA (OXOID), NA(OXOID) and NB(OXOID) at pH of 5.5, 5.7, 8, and 9 on NA(OXOID) mixed with Sodium Chloride(NaCl) ranging from 2% - 9%. and then incubated for 24 hrs at a temperature of 37°C. This was to get as wide a substrate base as possible and establish whether or not the strains are nutritionally fastidious. The dominant *Bacillus* strains were inoculated on NA (28 g/litre prepared according to the manufacturers' instructions) supplemented with 0.01g (0.04%), 0.05g(0.2%), 0.1g(0.4%), 0.5g(2%) and 1.0g(4%) quantities of 48-hrs, dried pellet and the finished product (kantong) samples.

*Inhibition of pathogenic microorganisms using agar well diffusion assay*

Each test *Bacillu*s strain stabbed in NA for preservation was revived in NB. A loopful was spread on NA and incubated at 37ºC for 24hrs in order to get distinct pure colonies. A pure colony from each of the agar plates was inoculated into 5 ml NB and incubated for 18hrs at 37ºC. The NB culture at the stationary phase of growth was centrifuged at 5000 x g for 10minutes in order to obtain a cell-free

supernatant (CFS). The CFS was filtered through 0.22 µl membrane filter, using a 10 ml syringe as siphon, to remove any cell left in the supernatant. The filtrate was then treated with 5 mg /ml catalase to eliminate Hydrogen Perioxide. About 10 – 15 ml of Brain Heart Infusion Agar [BHIA](OXOID) inoculated with $10^6$ CFU /ml of indicator microorganism was poured into a Petri dish and allowed to solidify (Kabore *et al*., 2011). Wells were cut with a sterile 6mm cork borer in the agar and filled with 50 µl of CFS. The plate was incubated for 24 hrs at 37ºC. The inhibition zone diameter (in mm) was measured with a pair of dividers and metre rule. The experiment was performed in triplicate and repeated twice .

*Inhibition of pathogenic microorganisms using agar spot testing*

The agar spot test as described by Kabore *et al.* (2011) was carried out. A 100 µl cell suspension of indicator microorganism was mixed with about 10 – 15 ml BHIA(OXOID) in a Petri dish and allowed to set. The cells from the stock used for the agar well diffusion test were suspended in 5 ml of sterile saline solution containing 8.5g/l NaCl and 1.5 g/l Bactopeptone (DIFCO), and the pH adjusted to 7.0. The number of microorganisms was estimated by microscopy using a counting chamber (Neubauer) and dilutions were made in sterile saline (0.85%, w/v NaCl) to obtain a rate of inoculation of 105–106 cells /ml. Approximately 50 µl of the test Bacillus strain was spotted on the surface of the BHIA and the dish incubated at 37ºC for 24 hrs. The presence of a clear zone around the spot indicated inhibition and the diameter was measured as before in millimetres. The experiment was performed in triplicate and repeated twice.

*Statistical analysis*

Means and standard deviations were determined for microbial counts. The means were analyzed using one-way Analysis of Variance (ANOVA) and Tukey test was used to compare the means when a significant variation was established by ANOVA at the significance level ($p = 0.05$).

## Results

*Growth temperature and pH*

The twelve strains of *Bacillus* spp. proliferated within different temperature ranges. Four isolates, *B. circulans, B. coagulans, B. subtilis* and *B. licheniformis* possessed the ability to endure a maximum growth temperature of 55°C, followed by *B. amyloliquefaciens, B. safensis* and *B. pumilus* with maximum of 50°C. All others could only endure a maximum growth temperature of 45°C (Table 1).

Table 1. Growth temperature range and substrate utilization preference of selected strains of *Bacillus* spp. isolated from kantong production

| *Bacillus* spp. | Temp (°C) | Substrates Utilized |
|---|---|---|
| *B. amyloliquefaciens* | 30-50 | NB, NA, PDA, TSA |
| *B. safensis* | 15-50 | NB, NA, PDA, TSA |
| *B. altitudinis* | 20-45 | NB, NA, PDA, TSA |
| *B. thuringiensis* | 10-45 | NB, NA, PDA, TSA |
| *B. pumilus* | 25-50 | NB, NA, PDA, TSA |
| *B. megaterium* | 25-45 | NB, NA, PDA, TSA |
| *B. cereus* | 15-45 | NB, NA, PDA, TSA, MCA |
| *B. circulans* | 20-55 | NB, NA, PDA, TSA |
| *B. coagulans* | 25-55 | NB, NA, PDA, TSA |
| *B. firmus* | 20-45 | NB, NA, PDA, TSA |
| *B. subtilis* | 15-55 | NB, NA, PDA, TSA |
| *B. licheniformis* | 25-55 | NB, NA, PDA, TSA |

NA – Nutrient Agar, NB – Nutrient Broth, PDA – Potato Dextrose Agar, TSA – Tryptone Soya Agar, MCA – MacConkey Agar

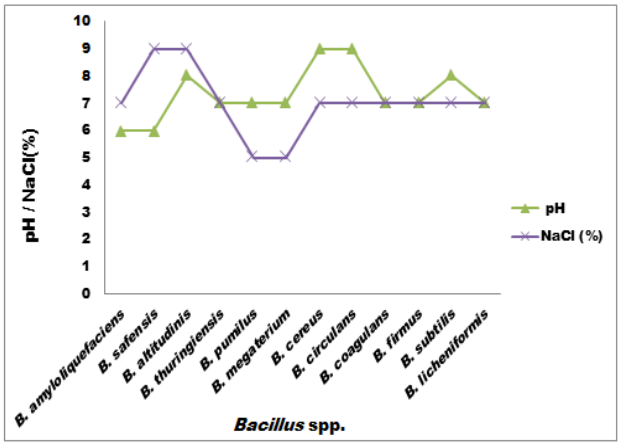


Figure 1. Maximum growth pH and salt tolerance of selected strains of *Bacillus* spp. isolated from kantong

Eight isolates (*B. amyloliquefaciens, B. safensis, B. thuringiensis, B. pumilus, B. megaterium, B. coagulans, B. firmus* and *B. licheniformis*) could survive acidic to neutral conditions of growth. The four others (*B. cereus, B. circulans, B. subtilis* and *B. altitudinis*) however, could endure acidic to alkaline conditions of growth (Figure 1).

Table 2. Counts of predominant *Bacillus* spp. on kantong-formulated media

| % of *kantong* | 48-hour | Dried Pellet | Final Product |
|---|---|---|---|
| | | ***B. amyloliquefaciens*(O1)** | |
| 0.04 | [a1.4±0.22] × $10^3$ | [a1.2±0.66] × $10^4$ | [a3.5±0.45] x $10^5$ |
| 0.2 | [a1.9±0.36] × $10^4$ | [a1.3±0.31] × $10^5$ | [a3.9±0.18] × $10^5$ |
| 0.4 | [a2.9±0.23] × $10^5$ | [a2.9±0.30] × $10^6$ | [a4.1±0.78] × $10^6$ |
| 2.0 | [a3.1±0.28] × $10^6$ | [a3.1±1.76] × $10^7$ | [b5.0±0.32] × $10^7$ |
| 4.0 | [b3.5±1.35] × $10^7$ | [a3.0±1.01] × $10^8$ | [c5.4±0.59] × $10^7$ |
| | | ***B. safensis* (T9)** | |
| 0.04 | [a1.6±0.51] × $10^3$ | [a2.4±0.64] × $10^4$ | [a3.5±0.31] × $10^4$ |
| 0.2 | [a1.8±0.27] × $10^4$ | [a1.3±0.07] × $10^5$ | [a3.9±0.24] × $10^5$ |
| 0.4 | [b3.0±0.78] × $10^5$ | [a2.9±0.57] × $10^6$ | [a4.1±0.27] × $10^6$ |
| 2.0 | [c3.1±0.37] × $10^6$ | [b3.9±1.02] × $10^7$ | [a5.0±0.80] × $10^6$ |
| 4.0 | [c3.5±0.65] × $10^7$ | [a4.0±0.44] × $10^7$ | [a5.2±0.87] × $10^6$ |
| | | ***B. altitudinis* (R2)** | |
| 0.04 | [a1.5±1.14] × $10^3$ | [a1.2±0.43] × $10^4$ | [a2.0±0.36] × $10^4$ |
| 0.2 | [a2.9±0.91] × $10^4$ | [b3.3±0.37] × $10^5$ | [a3.1±0.27] × $10^5$ |
| 0.4 | [a2.7±0.48] × $10^5$ | [a3.7±0.40] × $10^6$ | [a2.1±0.55] × $10^6$ |
| 2.0 | [a3.1±0.17] × $10^6$ | [a4.1±0.34] × $10^7$ | [a2.0±0.72] × $10^7$ |
| 4.0 | [b5.4±0.46] × $10^7$ | [c6.1±0.39] × $10^8$ | [c3.4±0.43] × $10^8$ |

[1]Values are means of triplicate determinations from three independent trials; ± = Standard deviations (SD)
[2]Means with same letters as superscripts in a column are not significantly different (P = 0.05).

*Salt tolerance and substrate utilization by isolates*

*B. safensis* and *B. altitudinis* exhibited the greatest tolerance of 9% for NaCl, followed by *B. amyloliquefaciens, B. thuringiensis, B. cereus, B. circulans, B. coagulans, B. firmus, B. subtilis* and *B. licheniformis*, with a maximum tolerance value of 7%. *B. pumilus* and *B. megaterium* could only tolerate up to 5% of NaCl (Figure 1). All the isolates grew massively on Nutrient Broth, Nutrient Agar, Potato Dextrose Agar and Tryptone Soya Agar. *B. cereus* utilized MacConkey Agar as an additional substrate (Table 1).

*Growth on kantong – formulated media*

Table 2 shows the counts of the three most dominant strains of *Bacillus* spp. on the kantong-formulated media involving the 48-hrs sample, dried pellet and the final product. Counts of *B. amyloliquefaciens* (O1) ranged between$10^3$ and $10^7$CFU /g; $10^4$ and $10^8$CFU /g and $10^5$ to $10^7$ CFU /g in the media formulated with the 48-hrs sample, dried pellet and final product, respectively. For *B. safensis* (T9), counts ranged from $10^3$ to 107CFU /g for the 48-hrs sample; $10^4$ to $10^7$CFU /g for the dried pellet and $10^4$ to $10^6$CFU /g for the final product. The ranges were $10^3$ to $10^7$CFU /g; $10^4$ to $10^8$CFU /g and$10^4$ to $10^8$CFU /g for the three media types respectively inoculated with *B. altitudinis* ($R^2$). Different amounts of the samples were taken at 48 hrs but there was no significant difference (p = 0.1915) for the number of isolates of the various *Bacillus* spp., however the number of isolates from the different sample quantities varied significantly (p = 0.0465) irrespective of the type of *Bacillus* species. No significant difference (p = 0.4162), was observed between the number of isolates identified on the dried pellets. The final product did not show any significant different (p = 0.4141) for the *Bacillus* spp. on the different quantities.

Table 3. Inhibition of pathogenic microorganisms by strains of dominant *Bacillus* spp. isolated from different stages of kantong production using the agar well diffusion assay

| | Isolate | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *B. amyloliquefaciens* | | | *B. altitudinis* | | | *B. safensis* | | | *B. subtilis* | | |
| **Pathogenic organism** | $O_1$ | $O_2$ | $O_4$ | $I_3$ | $R_{10}$ | $L_{10}$ | $T_9$ | $S_5$ | $J_9$ | $K_2$ | $N_6$ | $Q_7$ |
| *E. coli* (DSM 682) | +++ | ++ | - | - | - | ++ | - | - | - | ++ | ++ | - |
| *E. coli* (i9) | - | + | - | - | - | - | ++ | +++ | - | ++ | - | ++ |
| *Sal. typhimurium* | - | ++ | ++ | - | - | ++ | - | - | - | - | +++ | - |
| *K. pneumoniae* (g8i) | - | - | - | - | - | - | - | - | - | - | - | - |
| *P. aeruginosa* (DSM 939) | + | - | - | ++ | - | - | - | - | - | +++ | - | - |
| *Sh. flexneri* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Staph. aureus* | + | ++ | + | - | ++ | ++ | ++ | + | ++ | ++ | + | ++ |
| *A. baumannii* (t3) | - | - | - | - | +++ | - | +++ | - | - | - | - | - |
| *E. faecium* (e5i) | + | ++ | + | - | +++ | - | + | - | - | - | - | ++ |
| *E. cloacae* (h7) | ++ | - | - | - | - | - | - | ++ | ++ | ++ | - | - |
| *Pr. vulgaris* | +++ | - | - | + | + | + | - | - | - | ++ | - | ++ |
| *L. monocytogenes* | - | - | - | - | - | +++ | - | - | ++ | - | - | - |

-: no inhibition; +: 1mm < inhibition zone < 3mm; ++: 3mm ≤ inhibition zone < 5mm; +++: inhibition zone ≥ 5mm

*Inhibition of pathogenic microorganisms using agar well diffusion assay*

As shown in Table 3, ten out of the twelve test pathogenic organisms were susceptible to all twelve strains of the selected *Bacillus* spp. (*B. amyloliquefaciens, B. altitudinis, B. safensis* and *B. subtilis*) at varying degrees. Strain O1 of *B. amyloliquefaciens* and strain K2 of *B. subtilis* each inhibited six of the ten susceptible test organisms. Strain O2 of *B. amyloliquefaciens* and strain L10 of *B. altitudinis* also showed varying degrees of inhibitory activity against five test pathogenic organisms. *B. altitudinis* strain I3 was the least active, inhibiting only *Pr. vulgaris* and *P. aeruginosa*. *Staph. aureus* was the most susceptible pathogenic organism, being inhibited by all the strains except I3. None of the *Bacillus* strains was able to inhibit *K. pneumoniae* or *Sh. flexneri*. The largest inhibition zone diameter of ≥ 5mm was exhibited by *B. amyloliquefaciens* strain O1 against *E. coli* and *Pr. vulgaris; B. altitudinis* strain R10 against *A. baumannii* and *Ent. faecium*; *B. altitudinis* strain L10 against *L. monocytogenes*; *B. safensis* strainT9 against *A. baumannii*; *B. safensis* strain S5 against *E. coli*; *B. subtilis* strain K2 against *P. aeruginosa*, and *B. subtilis* strain N6 against *Sal. typhimurium.*

*Inhibition of pathogenic microorganisms using agar spot testing*

Similar to the results of the agar well diffusion assay, all twelve strains of the selected *Bacillus* spp. (*B. amyloliquefaciens, B. altitudinis, B. safensis* and *B. subtilis*) were able to inhibit ten out of the twelve test pathogenic organisms at varying degrees as shown in Table 4. Strains O1 of *B. amyloliquefaciens* and N6 of *B. subtilis* variously each inhibited seven of the ten susceptible test organisms while *B. amyloliquefaciens* strain O2 and *B. subtilis* strains K2 and Q7 also showed varying degrees of inhibitory activity against six test pathogenic organisms. *Staph. typhimurium* was inhibited by all the strains with *K. pneumoniae* and *A. baumannii* being susceptible to just two strains each. The largest inhibition zone diameter of ≥ 5mm was exhibited by *B. altitudinis* strain R10 against *E. faecium* and *B. subtilis* strain N6 against *Sh. flexneri.*

## Discussion

The process of fermenting *C. pentandra* seeds to produce kantong was studied to determine the microorganisms involved in the fermentation as well as the enzymatic activities involved. Lactic acid bacteria, yeasts and *Bacillus* spp. were the predominant

Table 4. Inhibition of pathogenic microorganisms by strains of dominant *Bacillus* spp. isolated from different stages of kantong production using the agar spot test

| | Isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *B. amyloliquefaciens* | | | *B. altitudinis* | | | *B. safensis* | | | *B. subtilis* | | |
| **Pathogenic organism** | $O_1$ | $O_2$ | $O_4$ | $I_3$ | $R_{10}$ | $L_{10}$ | $T_9$ | $S_5$ | $J_9$ | $K_2$ | $N_6$ | $Q_7$ |
| *E. coli* (DSM 682) | + | ++ | - | - | - | ++ | - | - | - | ++ | ++ | - |
| *E. coli (i9)* | - | + | - | - | - | - | ++ | + | - | ++ | - | ++ |
| *Sal. typhimurium* | + | ++ | ++ | + | + | ++ | + | + | + | + | ++ | ++ |
| *K. pneumonia* (g8i) | - | - | - | - | + | - | - | - | - | - | - | ++ |
| *P. aeruginosa* (DSM 939) | + | + | - | ++ | - | - | - | - | - | + | ++ | - |
| *Sh. flexneri* | - | - | - | + | - | - | - | - | + | - | +++ | - |
| *Staph. aureus* | + | + | + | - | - | - | ++ | + | + | ++ | + | ++ |
| *A. baumannii* (t3) | - | - | - | - | + | - | + | - | - | - | - | - |
| *E. faecium* (e5i) | + | ++ | + | - | +++ | - | + | - | - | - | - | ++ |
| *E. cloacae* (h7) | ++ | - | - | - | - | - | - | ++ | ++ | ++ | ++ | - |
| *Pr. vulgaris* | + | - | - | + | + | + | - | - | - | ++ | - | ++ |
| *L. monocytogenes* | - | - | + | - | - | + | - | - | ++ | - | + | - |

-: no inhibition; +: 1mm < inhibition zone < 3mm; ++: 3mm ≤ inhibition zone < 5mm; +++: inhibition zone ≥ 5mm

microorganisms identified and characterized by traditional microbiological methods as described by Kpikpi (2006). These predominant microorganisms have been reported to be found in most fermenting oil seeds (Odunfa and Oyewole, 1986; Ouoba *et al*., 2003) and they are also known to be highly proteolytic (Kpikpi, 2006). The predominant *Bacillus s*pp. which were spread variously along the eleven stages of kantong production were *B. amyloliquefaciens, B. safensis, B. altitudinis, B. thuringiensis, B. pumilus, B. megaterium, B. cereus, B. circulans, B. coagulans, B. firmus, B. subtilis,* and *B. licheniformis*.

The current study focused on some technological properties of the *Bacillus* spp. isolated from the various stages of kantong production. Isolates possessed the ability to endure growth temperatures spanning a wide range of 10°C to 55°C, an indication that these *Bacillus* spp. can be used as starter cultures for fermentation and production of kantong under a variety of climatic conditions.

The combination of high pH and free ammonia along with very rapid growth of the microorganisms at relatively high temperature above 40°C make it very difficult for other microorganisms that might spoil the product to grow (Steinkraus, 1995, 1997). B. amyloliquefaciens and *B. safensis* could do well in only an acidic environment; *B. thuringiensis, B. pumilus, B. megaterium, B. firmus,* and *B. licheniformis* could survive acidic to neutral conditions of growth, while *B. altitudinis, B. cereus, B. circulans* and *B. subtilis* could endure acidic to alkaline conditions of growth. This ability of most of the isolates to proliferate under various pH environments again supports the feasibility of using them as combined starter cultures for the production of kantong. Some studies have shown that fermentation of proteinaceous oil seeds are mainly alkaline fermentations with Bacillus spp. being the predominant microorganisms involved in the fermentation process (Odunfa, 1985; Ikenebomeh, 1989). Alkaline fermentations are generally considered safe (Steinkraus, 1995, 1997).

*B. amyloliquefaciens* which was not initially isolated from the final product, proliferated massively on kantong-formulated media at 48hrs, in the dried pellet and final product, just like *B. safensis* and *B. altitudinis* reaching counts of $10^7$ - $10^8$ CFU /g when the final product was incorporated into a basic dehydrated medium. The high counts registered by these predominant *Bacillus* spp. is an indication that kantong can serve as a formidable substitute for synthetic substrates in the propagation of the isolates as starter cultures for commercial scale kantong production.

All isolates variously exhibited inhibitory activity against ten out of the twelve pathogenic organisms. This is a clear indication that *Bacillus* spp. might

produce constitutively, antimicrobial compounds such as subtilin, as suggested earlier by Ouoba *et al.* (2007). However, all the three *B. subtilis* strains and two of each of *B. amyloliquefaciens* and *B. altitudinis* inhibited growth of majority of test pathogenic organisms. *Bacillus subtilis* strains were reported by Kuipers *et al.* (1992), Klein *et al.* (1993), Paik *et al.* (1998), Stein *et al*. (2004) and Kabore *et al.* (2011) to produce proteinaceous antibiotics such as subtilin, subtilosin, sublancin which are mostly active against Gram-positive bacteria, while a few may be active against Gram-negative bacteria (Katz and Demain. 1987; Bechard *et al.* 1998; Stover and Driks, 1999). It has also been reported that *B. subtilis* isolated from soumbala produces peptides, lipopeptide and protein substances which inhibit fungi (N'dir *et al*., 1994; Tosato *et al*., 1997). It's exceptional antimicrobial activity was therefore not surprising. According to Gutowski-Eckel *et al.* (1994), Stover and Driks (1999) and Prescott *et al.* (2002), *Bacillus* isolates produced different antimicrobial compounds during the logarithmic and /or stationary growth phase as well as in the sporulated form, justifying the generally different degrees of inhibitory activity exhibited by the various strains.

The inhibition of pathogenic microorganisms such as *E. coli, K. pneumoniae, Ac. baumannii, Ent. cloaclae, Sal. typhimurium, P. aeruginosa, Pr. vulgaris, Ent. faecium* and *Staph. aureus* by the Bacillus strains is a positive development as it will enhance the shelf-life and purity of kantong. *E.coli* (i9) existed with the five strains of *Bacillus* in kantong but these were found to inhibit its growth during the inhibitory tests. It could be possible that in kantong, extraneous factors like organic material and competition from other organisms prevented direct contact between it and its protagonist, thus reducing the inhibitory effect.

According to Sanni (1993) and Hounhouigan *et al*.(1999), the use of starter cultures containing these microorganisms to ferment cereal foods would be an appropriate way to control the fermentation process and to overcome problems of variation in organoleptic characteristics and thus, in product quality encountered in the natural fermentation. The production of kantong and most of the fermented foods in Africa depends on uncontrolled fermentation with sometimes inherent hygienic, nutritional and organoleptic defects (Odunfa, 1981; Odunfa and Adewuyi, 1985; Antai and Ibrahim, 1986; N'dir *et al*., 1997). This means that when strains of *B. subtilis, B. amyloliquefaciens* and *B. altitudinis* are considered for starter culture development, singly or in combination, they might prevent the occurrence of pathogenic microorganisms during the fermentation of *C. pentandra* seeds, leading to a hygienic, nutritive and reliable production of kantong as a food condiment. The variability among the isolates in these technological properties could be explained by differences observed in their genotypes as described by Ouoba *et al.* (2004).

## Conclusion

Our study has established that a wide variety of strains of *B. amyloliquefaciens, B. safensis, B. altitudinis, B. thuringiensis, B. pumilus, B. megaterium, B. cereus, B. circulans, B. coagulans, B. firmus, B. subtilis,* and *B. licheniformis* were the predominant species found to be responsible for fermenting *C. pentandra* seeds during kantong production. Strains of all these selected predominant *Bacillus* spp. possessed desirable proliferation temperature and pH, and substrate utilization preferences which make them candidates for starter culture development to produce kantong of acceptable quality and thus lead to commercialization of the product. In addition, strains of *B. subtilis, B. amyloliquefaciens* and *B. altitudinis* possessed the capability to effectively inhibit the growth of some pathogenic microorganisms which can cause spoilage of kantong, thereby shortening its shelf-life as well as rendering it unsafe for consumption.

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