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Full Length Research Paper

Esterase and protease activities of *Bacillus* spp. from *afitin*, *iru* and *sonru*; three African locust bean (*Parkia biglobosa*) condiments from Benin

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Fifty strains of *Bacillus* spp. comprising *Bacillus subtilis* and *Bacillus licheniformis* previously isolated from *afitin*, *iru* and *sonru* were examined for esterase and protease activities. The electrophoretic profiles of fermented African locust bean protein (ALBP), using strains presenting the highest protease activities in casein agar, were analyzed by SDS-PAGE to select strains with good ability to be used as starter cultures. All the *Bacillus* spp. tested showed esterase activity against tributyrin with high variability among strains. Strains showing the highest esterase activities were *B. subtilis,* primarily isolated from *iru*, *sonru* and lastly in *afitin*. Only 62% (31/50) of the *Bacillus* strains tested showed perceptible, but highly variable protease activity in casein agar. *Bacillus* strains showing the highest protease activities comprised strains of *B. subtilis* isolated from *afitin* and *iru and* strains of *B. licheniformis* isolated from *iru* and *sonru*. A *B. subtilis* strain isolated from *afitin* showed high esterase as well as high protease activity. The electrophoretic profile by SDS-PAGE of ALBP fermented by the *Bacillus* spp. having the highest protease activities showed degradation products with a wide range of molecular size between 4 and 250 kDa. Variability of the characteristics of the *Bacillus* spp. tested give new opportunities for their use as starter culture for products development.

Key words: *Bacillus subtilis*, *bacillus licheniformis*, fermentation, esterase and protease activity, SDS-PAGE.

INTRODUCTION

Fermentation of African locust bean (*Parkia biglobosa*) is the key process of afitin, iru and sonru for which Bacillus spp. were found to be the dominant microorganisms (Azokpota et al., 2005a) as also in the case of dawadawa (Odunfa, 1985; Odunfa 1986), soumbala (Diawara et al., 1992; Beaumont, 2002) and netetu (N'dir et al 1994) Lipolysis and proteolysis are very important for the quality of African locust bean-based condiments fermented by Bacillus spp. (Fetuga et al., 1974; Odunfa and Adesomoju, 1986; Aderibigbe and Odunfa 1988; Wang and Fung, 1996; Ouoba et al., 2003a). Proteolysis has been reported as the main metabolic activity during the fermentation of African locust bean (Odunfa, 1985; Aderibigbe and Odunfa, 1988; Aderibigbe and Odunfa, 1990; Allagheny et al., 1996). Metabolic properties of Bacillus through the degradation of proteins contribute directly or indirectly to the development of the texture and flavour of the fermented products (Odunfa, 1985; Wand and Fung, 1996; Ouoba et al., 2003a). The proteolytic system of Bacillus may also contribute to the liberation of bioactive peptides which could play a significant role in the enzymatic process involved during the fermentation.

Specifically, peptides may also have inhibitory activitiestowards microbial proteolytic enzymes in food spoilage (Meisel and Bockemann, 1999).

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Although lipids are a major constituent of the African locust bean (Ouoba et al., 2003b), lipolytic activity has been reported to be fairly low and has been attributed to Staphylococcus species found in the fermenting seeds (Odunfa, 1985; Ouoba et al., 2003b). Nevertheless, lipolytic activities of Bacillus spp. are expected to have an impact on the organoleptic quality of products (N'dir et al., 1997;).

Commonly employed techniques for detecting lipolytic and proteolytic activity of micro-organisms are agar diffusion tests using different substrates (Vuillemard et al., 1985; Vuillemard and Amiot, 1985; Larsen et al., 1998; Larsen and Jensen, 1999). Lipases are diverse groups of enzymes that catalyze the hydrolysis of ester bonds in triacylglycerides, showing activity on a great variety of substrates. Agar plates supplemented with tributyrin or olive are often recommended to examine esterase and lipolytic activity respectively in Bacillus species (Ruiz et al., 2002). Lipases differ from esterase enzymes since the latter cleave only water-soluble esters such triacglycerol (Belitz and Grosch, 1987).

In a previous study, the dominant species involved in the fermentation of African locust bean to produce afitin, iru and sonru were found to be strains of the B. subtilis group (Azokpota et al., 2005b). The purpose of this work was to examine the esterase and proteolytic activities of strains of B. subilis and B. licheniformis previously isolated from afitin, iru and sonru. The tests were performed using the agar diffusion tests in tributyrin agar for esterase activity and casein agar for proteolytic activity. Bacillus strains presenting highest proteolytic on casein agar were used to ferment African Locust Bean Proteins (ALBP) and the electrophoretic profiles of the ALBP products were determined by SDS-PAGE.

MATERIAL AND METHODS

Microorganisms

Forty (40) strains of *B. subtilis* and ten (10) strains of *B. licheniformis* previously isolated and identified from *afitin*, *iru* and *sonru* at different fermentation times (Azokpota et al., 2005b) were used.

Extraction of African locust bean proteins (ALBP)

The ALBP extract was obtained as described by Ouoba et al. (2003a). African locust beans were dehulled and grounded into powder. The lipids were removed by soxhlet method and the proteins extracted according to Berot and Davin (1985). The defatted powder (100 g) obtained was mixed with 1000 ml distilled water. The mixture was heated at 70°C under agitation for 1 h and centrifuged at 5000 x g for 10 min. After centrifugation, the pellet was washed with distilled water and freeze dried.

Preparation of inocula

The strains were cultured at 37°C for 18 h in Nutrient Agar (Remel, 454182, Bie and Berntsen, Rødovre, Denmark) and sub-cultured in 10 ml of Nutrient Broth (Difco 0003-17-8, Detroit, MI, USA) at 37°C under agitation for 18 h. The cultures were centrifuged (Sorvall, RC 5B centrifuge) at 5000 x g for 10 min and the pellet was resuspended in 5 ml of sterile diluent containing 8.5 g/l NaCl and 1.5 g/l Bactopectone (Difco 0118-17, MD 21152 USA), pH 7.0. The number of the microorganisms per ml was estimated by microscopy using a counting chamber (Olympus CH CO11, Japan). From this concentration of cells, dilutions were made in sterile diluents to obtain an inoculum rate from 10^3 to 10^4 cfu/ml for the tests.

Determination of esterase activity

A basal substrate containing 1.2% Bactoagar (Difco 0140, MD 21152 USA), 0.5% Bactopeptone (Difco 0118-17, MD 21152 USA) and 0.3% yeast extract (Difco 0127-17, MD 21152 USA), adjusted to pH 7.0 was autoclaved at 121°C for 20 min and 0.1% filter sterilized tributyrin (Sigma T-8626, Louis, Mo 63178 USA) was added after cooling to 60°C (Larsen and Jensen, 1999). The mixture was aseptically emulsified for 5 min at 13500 rpm using an Ultra Turrax emulsifier (Junke and Kunrel IKA, Labortechnic, Taufen, Germany). Ten (10) ml were distributed into sterile tubes. After solidification, 100 µl of inoculum for each *Bacillus* strain were added on the top of the agar in a tube. The tubes were incubated at 37°C for 72 h. A clearing zone indicated hydrolysis of tributyrin and the depth of the clearing zones was measured with ruler every 24 h. Trials were conducted in duplicate at three different occasions.

Determination of protease activity

Nutrient Agar (Remel, 454182, Bie and Berntsen, Rødovre, Denmark) was supplemented with 2% of casein (Sigma C-5890, St Louis, MO USA), autoclaved at 121°C for 20 min and distributed in Petri dishes. Wells of 8 mm diameter were made in the middle of the agar in Petri dishes after solidification, using a sterilized cork barer and 100 µl of inoculum from each *Bacillus* strain were transferred to the wells followed by incubation at 37°C for 72 h. At each 24 h of incubation, the clearing zone around each well was measured as an indicator of protease activity. Trials were conducted in duplicate at three different occasions.

Strains showing the highest protease activity in casein agar were selected for the fermentation of ALBP and the SDS-PAGE analysis. The electrophoretic profiles of ALBP fermented for 24, 48 and 72 h at 37 °C by the *Bacillus* strains selected were performed using SDS-PAGE as described by Ouoba et al. (2003a).

Statistical analyses

Statistical differences between mean values of the depth of clearing zones in tributyrin agar (for esterase activity) and the diameter in casein agar (for protease activity) were determined by analysis of variance and the general linear model procedures (SAS, release 6-12, Cary, NC, USA). Significance was accepted at $P< 0.05$ using Duncan's multiple range test.

RESULTS

All the *Bacillus* spp. tested showed esterase activity against tributyrin, which increased significantly with incubation time (Table 1). High variability was found among *Bacillus* strains, independently of the species and their origin, allowing their classification into 7 groups a to g, according to the esterase activity as expressed by

Table 1. Esterase activity of 50 *Bacillus* strains investigated by diffusion test on tributyrin agar after 3 days of incubation at 37°C.

No.	Code[1]	Species	Fermented condiments	Depth[2] of clearing zone (mm)		
				24 h	48 h	72 h
1	Y18H4	*B. subtilis*	*Iru*	13.9^{a}	20.9^{a}	21.5^{a}
2	Y24H5	*B. subtilis*	*Iru*	12.5^{b}	19.8^{b}	20.5^{b}
3	Ba12H16	*B. subtilis*	*Sonru*	11.4^{c}	19.6^{b}	20.3^{b}
4	Ba12H15	*B. subtilis*	*Sonru*	11.1^{c}	17.0^{c}	20.2^{b}
5	F12H9	*B. subtilis*	*Afitin*	11.1^{c}	17.0^{c}	18.8^{c}
6	F0H1	*B. subtilis*	*Afitin*	10.5^{cd}	17.0^{c}	18.7^{c}
7	F24H10	*B. subtilis*	*Afitin*	10.5^{cd}	15.2^{d}	18.6^{c}
8	Y0H2	*B. subtilis*	*Iru*	10.4^{cd}	15.2^{d}	18.5^{c}
9	Ba24H10	*B. licheniformis*	*Sonru*	10.4^{cd}	15.2^{d}	18.5^{c}
10	F24H3	*B. subtilis*	*Afitin*	10.4^{cd}	14.2^{e}	17.7^{d}
11	Ba48H5	*B. licheniformis*	*Sonru*	10.4^{cd}	14.2^{e}	17.6^{d}
12	Ba24H14	*B. licheniformis*	*Sonru*	10.4^{cd}	14.2^{e}	17.4^{d}
13	Y18H6	*B. subtilis*	*Iru*	10.4^{cd}	14.2^{e}	15.0^{e}
14	F0H7	*B. subtilis*	*Afitin*	10.4^{cd}	14.1^{e}	15.0^{e}
15	Y18H5	*B. subtilis*	*Iru*	10.4^{cd}	14.1^{e}	15.0^{e}
16	F6H10	*B. subtilis*	*Afitin*	10.4^{cd}	14.1^{e}	15.0^{e}
17	F0H2	*B. subtilis*	*Afitin*	10.4^{cd}	14.0^{e}	14.4^{f}
18	F6H9	*B. subtilis*	*Afitin*	10.4^{cd}	14.0^{e}	14.4^{f}
19	Ba18H12	*B. subtilis*	*Sonru*	10.4^{cd}	14.0^{e}	14.4^{f}
20	F12H8	*B. subtilis*	*Iru*	10.4^{cd}	14.0^{e}	14.4^{f}
21	Ba0H10	*B. subtilis*	*Sonru*	10.2^{cd}	14.0^{e}	14.4^{f}
22	Y36H10	*B. licheniformis*	*Iru*	10.2^{cd}	14.0^{e}	14.4^{f}
23	Ba18H13	*B. subtilis*	*Sonru*	10.2^{cd}	14.0^{e}	14.4^{f}
24	Ba24H5	*B. subtilis*	*Sonru*	10.2^{cd}	14.0^{e}	14.2^{f}
25	Ba18H16	*B. subtilis*	*Sonru*	10.2^{cd}	14.0^{e}	14.2^{f}
26	Ba0H11	*B. subtilis*	*Sonru*	10.2^{cd}	14.0^{e}	14.2^{f}
27	Y6H4	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.2^{f}
28	F6H4	*B. subtilis*	*Afitin*	10.2^{cd}	14.0^{e}	14.1^{f}
29	F12H7	*B. subtilis*	*Afitin*	10.2^{cd}	14.0^{e}	14.1^{f}
30	Y36H5	*B. licheniformis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
31	Y24H9	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
32	Y6H2	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
33	Y36H9	*B. licheniformis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
34	Y12H4	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
35	F18H6	*B. subtilis*	*Afitin*	10.2^{cd}	14.0^{e}	14.1^{f}
36	Y12H2	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
37	Y0H3	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.1^{f}
38	F18H10	*B. subtilis*	*Afitin*	10.2^{cd}	14.0^{e}	14.1^{f}
39	Y12H1	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.0^{f}
40	Y24H10	*B. subtilis*	*Iru*	10.2^{cd}	14.0^{e}	14.0^{f}
41	Ba24H9	*B. licheniformis*	*Sonru*	10.2^{cd}	14.0^{e}	14.0^{f}
42	Y48H5	*B.licheniformis*	*Iru*	10.2^{cd}	14.0^{e}	14.0^{f}
42	Y36H4	*B. licheniformis*	*Iru*	10.2^{cd}	14.0^{e}	14.0^{f}
44	Ba0H5	*B. subtilis*	*Sonru*	09.4^{d}	14.0^{e}	14.0^{f}
45	Y48H2	*B. subtilis*	*Iru*	09.4^{d}	14.0^{e}	14.0^{f}
46	Ba18H14	*B. subtilis*	*Sonru*	09.2^{d}	14.0^{e}	14.0^{f}
47	F18H5	*B. subtilis*	*Afitin*	09.2^{d}	13.0^{f}	14.0^{f}
48	F24H9	*B. subtilis*	*Afitin*	09.2^{d}	12.1^{g}	14.0^{f}
49	Y48H9	*B. licheniformis*	*Iru*	08.3^{e}	12.1^{g}	12.4^{g}
50	Ba12H14	*B. subtilis*	*Sonru*	08.0^{e}	12.0^{g}	12.0^{g}

[1]F: *afitin*; Y: *iru*; Ba: *sonru* [2]The strains are listed in order of decreasing esterase activity. Values are means of triple determinations carried out on two different occasions. Means with the different letter (s) in a column are significantly different (p< 0. 05).

the depth of clearing zones at 72 h of fermentation. Strains showing the highest esterase activity in the first three groups a to c were *Bacillus subtilis* primarily isolated from *iru* (Y18H4 and Y24H5), from *sonru* (Ba12H16 and Ba12H15) and lastly from *afitin* (F12H9, F0H1, F24H10).

Table 2. Protease activity of 50 *Bacillus* strains investigated by diffusion test on casein agar after 3 days of incubation at 37°C.

No.	Code[1]	Species	Fermented condiments	Diameters[2] of clearing zone (mm)		
				24 h	48 h	72 h
1	Y48H5	*B.licheniformis*	*Iru*	35.5[a]	42.7[a]	45.5[a]
2	F24H10	*B.subtilis*	*Afitin*	35.2[a]	42.7[a]	45.4[a]
3	F18H5	*B.subtilis*	*Afitin*	35.2[a]	42.5[a]	45.4[a]
4	Y48H2	*B.subtilis*	*Iru*	35.2[a]	42.5[a]	45.2[a]
5	Y36H9	*B.licheniformis*	*Iru*	35.5[a]	42.5[a]	45.2[a]
6	Ba48H5	*B.licheniformis*	*Sonru*	33.7[b]	42.5[a]	45.2[a]
7	Y48H9	*B.licheniformis*	*Iru*	32.5[c]	41.0[b]	45.2[a]
8	F12H7	*B.subtilis*	*Afitin*	29.5[d]	40.1[c]	43.7[b]
9	Ba18H12	*B.subtilis*	*Sonru*	29.4[d]	40.1[c]	43.5[b]
10	Y18H5	*B.subtilis*	*Iru*	29.2[d]	40.1[c]	43.5[b]
11	Ba24H5	*B.subtilis*	*Sonru*	28.1[e]	39.2[d]	42.5[c]
12	Ba18H16	*B.subtilis*	*Sonru*	28.1[e]	38.2[e]	42.3[c]
13	Ba24H14	*B.licheniformis*	*Sonru*	28.1[e]	38.5[e]	42.3[c]
14	F18H10	*B.subtilis*	*Afitin*	28.1[e]	38.5[e]	42.3[c]
15	Ba24H9	*B.licheniformis*	*Sonru*	26.7[f]	38.5[e]	42.3[c]
16	Ba24H10	*B.licheniformis*	*Sonru*	26.7[f]	38.2[e]	42.3[c]
17	F18H6	*B.subtilis*	*Afitin*	26.7[f]	35.4[f]	42.3[c]
18	F24H3	*B.subtilis*	*Afitin*	26.7[f]	35.4[f]	40.6[d]
19	Y18H6	*B.subtilis*	*Iru*	26.6[f]	35.4[f]	40.5[d]
20	Y36H10	*B.licheniformis*	*Iru*	26.5[f]	35.4[f]	40.4[d]
21	Y12H4	*B.subtilis*	*Iru*	26.4[f]	35.2[f]	40.4[d]
22	F12H8	*B.subtilis*	*Afitin*	26.4[f]	35.2[f]	40.4[d]
23	Ba12H15	*B.subtilis*	*Sonru*	26.4[f]	35.2[f]	40.4[d]
24	Ba12H14	*B.subtilis*	*Sonru*	26.4[f]	35.2[f]	40.2[d]
25	Y18H4	*B.subtilis*	*Iru*	26.4[f]	35.2[f]	38.5[e]
26	Y12H2	*B.subtilis*	*Iru*	26.4[f]	35.2[f]	38.5[e]
27	Y6H2	*B.subtilis*	*Iru*	26.2[f]	35.1[f]	38.5[e]
28	F6H4	*B.subtilis*	*Afitin*	26.4[f]	35.1[f]	38.5[e]
29	F6H9	*B.subtilis*	*Afitin*	26.4[f]	35.1[f]	38.2[e]
30	Y6H4	*B.subtilis*	*Iru*	26.4[f]	35.1[f]	37.0[f]
31	Y12H1	*B.subtilis*	*Iru*	26.4[f]	35.1[f]	37.0[f]
32	F0H1	*B.subtilis*	*Afitin*	00.0[g]	00.0[g]	00.0[g]
33	F0H2	*B.subtilis*	*Afitin*	00.0[g]	00.0[g]	00.0[g]
34	F0H7	*B.subtilis*	*Afitin*	00.0[g]	00.0[g]	00.0[g]
35	F6H10	*B.subtilis*	*Afitin*	00.0[g]	00.0[g]	00.0[g]
36	Y24H10	*B.subtilis*	*Iru*	00.0[g]	00.0[g]	00.0[g]
37	F12H9	*B.subtilis*	*Afitin*	00.0[g]	00.0[g]	00.0[g]
38	Y36H5	*B.licheniformis*	*Iru*	00.0[g]	00.0[g]	00.0[g]
39	Ba0H10	*B.subtilis*	*Sonru*	00.0[g]	00.0[g]	00.0[g]
40	Ba0H5	*B.subtilis*	*Sonru*	00.0[g]	00.0[g]	00.0[g]
41	Ba12H16	*B.subtilis*	*Sonru*	00.0[g]	00.0[g]	00.0[g]
42	Ba0H11	*B.subtilis*	*Sonru*	00.0[g]	00.0[g]	00.0[g]
42	Y24H9	*B.subtilis*	*Iru*	00.0[g]	00.0[g]	00.0[g]
44	Ba18H14	*B.subtilis*	*Sonru*	00.0[g]	00.0[g]	00.0[g]
45	Y36H4	*B.licheniformis*	*Iru*	00.0[g]	00.0[g]	00.0[g]
46	F24H9	*B.subtilis*	*Afitin*	00.0[g]	00.0[g]	00.0[g]
47	Ba18H13	*B.subtilis*	*Sonru*	00.0[g]	00.0[g]	00.0[g]
48	Y0H2	*B.subtilis*	*Iru*	00.0[g]	00.0[g]	00.0[g]
49	Y0H3	*B.subtilis*	*Iru*	00.0[g]	00.0[g]	00.0[g]
50	Y24H5	*B.subtilis*	*Iru*	00.0[g]	00.0[g]	00.0[g]

[1]F: *afitin*; Y: *iru*; Ba: *sonru*

[2]The strains are listed in order of decreasing protease activity. Values are means of triple determinations carried out on two different occasions. Means with different letter (s) in a column are significantly different ($p < 0.05$).

The results of the diffusion test on casein agar are shown in Table 2. High variability was also found among the *Bacillus* for their protease activity at 72 h of incubation, independently of species and their origin, allowing a classification into 7 groups a to g. Only 6 groups a to f representing 62% (31/50) of the *Bacillus* strains tested showed protease activity in casein agar as expressed by the diameter of the clearing zone which

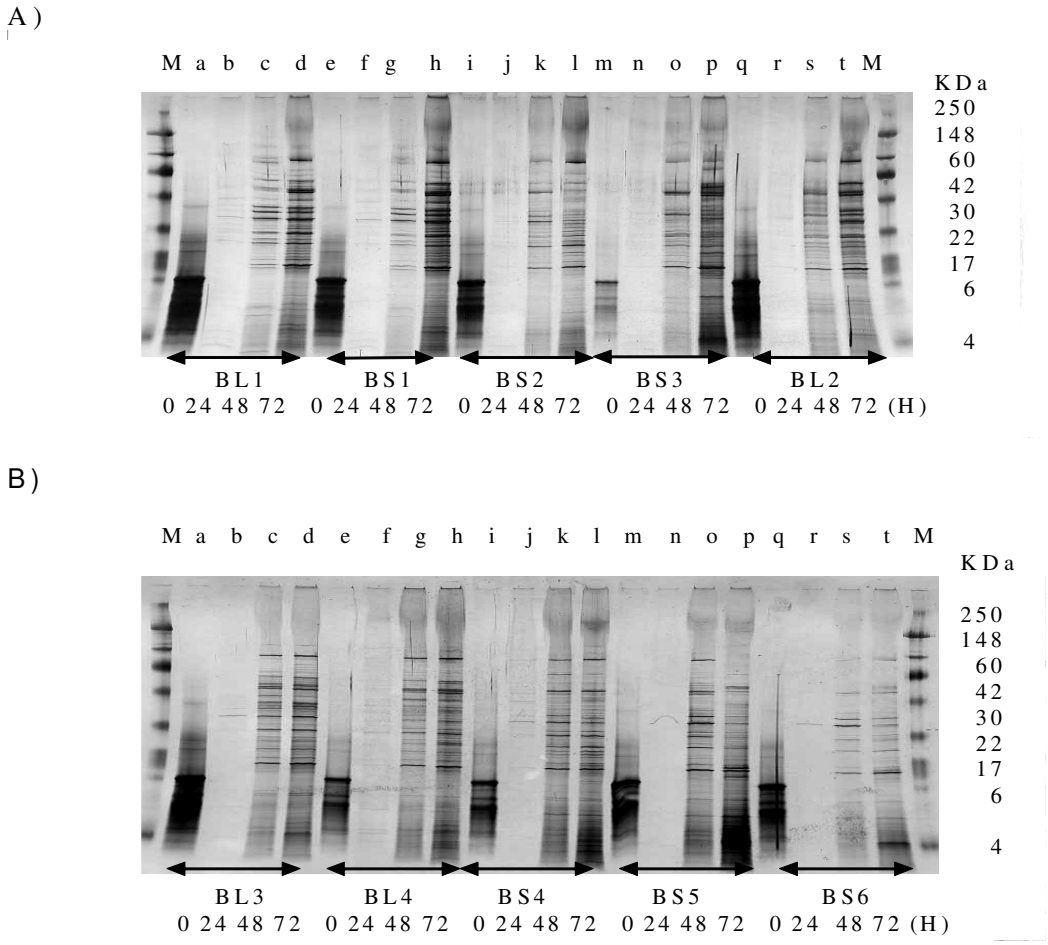

Figure 1. Elecrophoretic profiles by SDS-PAGE of non-fermented and fermented African locust bean proteins (ALBP) by six *B. subtilis* strains (BS1, BS2, BS3, BS4, BS5 and BS6) and four *B. licheniformis* strains (BL1, BL2, BL3 and BL4) isolated from *afitin*, *iru* and *sonru*. A and B): lanes a, e, i, m and q: electrophoretic profiles of unfermented ALBP (0 h of fermentation); lanes b, f, j, n and r: electrophoretic profiles of ALBP fermented by all the investigated strains at 24 h; A): Lanes (c, d), (g, h), (k, l), (o, p) and (s, t) : electrophoretic profiles of ALBP fermented by *Bacillus* strains BL1, BS1, BS2, BS3 and BL2, respectively. The two letters in brackets indicated the electrophoretic profiles for each strain at 48 and 72 h, respectively. B): Lanes (c, d), (g, h), (k, l), (o, p) and (s, t) : electrophoretic profiles of ALBP fermented by *Bacillus* strains BL3, BL4, BS4, BS5 and BS6, each at 48 and 72 h, respectively.

varied between 37 and 45.5 mm. They are composed of 57.5% (23/40) of the *B. subtilis* strains tested and 80% (8/10) of the *B. licheniformis* strains tested. *Bacillus* strains showing the highest protease activity, comprised strains of *B. subtilis* isolated from *afitin* (F12H0, F18H5) and iru (Y48H2) and strains of *B. licheniformis* isolated from *iru* (Y48H5, Y36H9, Y48H9) and *sonru* (Ba48H5). The seventh group comprising 42.5% (17/40) of the strains of *B. subtilis* and 20% (2/10) of the *B. licheniformis* isolates did not show any proteolytic activity in casein agar.

The strains of *Bacillus*, showing the highest proteolytic activity included six *B. subtilis* strains (three from *afitin*, two from *iru* and one from *sonru*): F24H10, F18H5, Y48H2, F12H7, Ba18H12, Y18H5 and four *B. licheniformis* strains (three from *iru* from *sonru*): Y48H5, Y36H9, Ba48H5, and Y48H9 (Table 2). They were designated BS1, BS2, BS3, BS4, BS5, BS6, BL1, BL2, BL3 and BL4, respectively and were selected to ferment ALBP prior to the SDS-PAGE analysis. As results of the proteins degradation, the pH values of the fermented ALBP were similar for all the strains tested and increased globally with the fermentation time from 5.2 at 0 h to 7.3 at 24 h, 8.4 at 48 h and 8.7 at 72 h, whereas the non inoculated ALBP used as control exhibited a lower pH value of 6.2 after the
same time (results not shown).

The electrophoretic profiles of the fermented ALBP are different from that of the nonfermented ALBP whatever the *Bacillus* strain tested, as shown in Figures 1A and 1B. The profiles exhibited differ according to the fermentation time for the same *Bacillus* strain. Compared to the

unfermented products (Figures 1A and 1B: lanes a, e, i; m, and q), it was seen that after 48 and 72 h of fermentation, many new bands appeared with molecular size varying between 4 and 250 kDa (Figures 1A and 1B: lanes c, d, g, h, k, l, o, p, s and t). The electrophoretic profiles of ALBP fermented by all the strains of *Bacillus* at 24 h of fermentation were not visible (Figures 1A and 1B: lanes: b, f, j, n, and r). At intraspecies level, at 48 h of fermentation, the electrophoretic profiles of ALBP fermented by *B. subtilis* strain BS1 (lane g, Figure 1A) and by *B. subtilis* strain BS2 (lane k) were similar and characterized by three central bands at the region of the 30 kDa which were absent for the profiles of ALBP fermented by the other *B. subtilis* strains BS3 (lane o, Figure 1A), BS4 (lane k), BS5 (lane o) and BS6 (lane s) (Figure 1B). At 72 h of fermentation, the profiles of ALBP fermented by all the *B. subtilis* strains (BS1, BS2, BS3, BS4, BS5 and BS6) were different.

The intraspecies profiles similarity was found also for the strains of *B. licheniformis* tested: at 48 h of fermentation, the ALBP fermented by the strains of *B. licheniformis* BL3 (lane c, Figure 1B) and BL4 (lane g, Figure 1B) showed similar electrophoretic profiles. In contrast, the profile of ALBP fermented by *B. licheniformis* strain BL1 (lane c) showing three central bands around 30 kDa, were different from the profile of ALBP fermented by *B. licheniformis* strain BL2 (lane s) where these bands were absent (Figure 1A). At 72 h of fermentation, the profiles of ALBP fermented by *B. licheniformis* strain BL1 (lane d, Figure 1A) and by *B. licheniformis* strain BL2 (lane t, Figure 1A) were similar but, both differ from the profiles of ALBP fermented by the strains of *B. licheniformis* BL3 (lane d) and BL4 (lane h) which were also similar (Figure 1B).

At interspecies level, differences were observed in general, but also some similarities were evident. As example, at 48 h of fermentation, the profile of ALBP fermented by *B. licheniformis* strain BL1 (lane c, Figure 1A) was similar to the profiles of ALBP fermented by the strains of *B. subtilis* BS1 (lane g) and BS2 (lane k) (Figure 1A). In addition, the ALBP fermented by the strains of *B. licheniformis* BL3 (lanes c, d), *B. licheniformis* strain BL4 (lanes g, h) and by *B. subtilis* strain BS4 (lanes k, l) (Figure 1B) were characterized by the same electrophoretic profiles at 48 and 72 h of fermentation.

DISCUSSION

All the fifty (50) strains of *Bacillus* investigated in the present study were able to hydrolyze tributyrin, presenting a pronounced variability in the esterase activity. The depth of the clearing zone increased with the fermentation time, due to the growth of the *Bacillus* strains as reported by Wang and Fung (1996) and Ouoba et al. (2003b). The highest activity was produced by a *B. subtilis* strain isolated from *iru*. But the esterase activity of the later was lower than that of the *Bacillus* spp. isolated from *soumbala* as reported by Ouoba et al. (2003b). It was reported that *Bacillus* spp. were characterized by two types of lipase LipA and LipB (Eggert et al., 2002) which possess specific activities (Ruiz et al., 2002). The ability of some strains to degrade more tributyrin than others might be due to the specificity of the enzymes they produced (Stepaniak et al., 1980; N'dir et al., 1997; Larsen and Jensen, 1999; Eggert et al., 2002). However, there is no information from the literature concerning the standard threshold values to be used as an effective tool to appreciate the good performance of esterase activity of strains isolated from some traditional fermented African locust bean-based condiments. An adequate lipolytic activity could probably be a good characteristic, since studies on the characterization of volatile aroma in *Bacillus*-fermented soybeans (Owens et al*.,* 1997; Peppard, 1999) and African locust bean (Odunfa and Adesomoju, 1986; Beaumont, 2002) showed the formation of desired aroma characteristics by free fatty acids resulting from the degradation of different substrates by the lipase activity of the strains,. However, aroma development should be kept at adequate level as too strong aroma of *afitin*, *iru* or *sonru* are generally a factor of rejection among consumers. Research is still needed to determine adequate free fatty acid and other aroma compounds contents compatible with *afitin*, *iru* or *sonru* acceptability among the consumers.

Protease activity of the strains in casein agar also increased, as the result of their metabolic activities during incubation as reported by Vuillemard and Amiot (1985), Griswold and Mackie (1997) and Ouoba et al. (2003a). Vuillemard et al. (1985) reported a threshold value of 20 mm for the diameter of clear zone of proteolytic bacteria in casein agar after 72 h of fermentation. On this basis, one can consider that 62% (31/50) of the investigated strains were highly proteolytic, even after 24 h of fermentation, with a quite similar distribution whatever the type of product: 60% (9/15) for *afitin* or *sonru* and 65% (13/20) for *iru*. In addition, all the *Bacillus* strains investigated in this study and showing high proteolytic activity in casein agar appeared to be more proteolytic than those identified in *soumbala* (Ouoba et al., 2003a). It was reported that the proteins hydrolysis depends on the specificity of the enzyme produced by the strain towards he three-dimensional structure of the proteins and their accessibility to the cut sites (Vuillemard and Amiot, 1985; Vuillemard et al., 1985). The difference in protease activity observed between strains could have a major influence on the characteristics of the fermented products (Chinivasagam et al., 1998; Mugula and Stepaniak, 2002). Strains with low protease activity could give less mature condiment with a longer shelf life, while strains with high protease activity will give a strong tasting condiment with shorter shelf life (Larsen et al., 1998; Martin et al., 2001; Rajmohan et al., 2002). *Afitin*, *iru* and *sonru* are characterized by the variability of proteolytic

activity. This characteristic could explain the variability of the quality of these condiments among producers (Azokpota et al., 2005a).

Increasing pH values of the fermented ALBP by the selected ten strains for SDS-PAGE with the fermentation time from acidity to the alkalinity indicated a degradation of proteins by the strains (Wang and Fung, 1996).

The profiles of ALBP fermented by all the strains at 24 h were not visible, for reasons that remains unexplained. Intraspecies and interspecies differences were exhibited by some *Bacillus* strains as found on the profiles of ALBP fermented by these strains at 48 and 72 h of fermentation. These differences could be linked to the specificity of the protease activity of the strains.

The similarities observed on the profiles of ALBP fermented by some *B. subtilis* and *B. licheniformis* strains isolated from different condiments indicated that these strains of the *Bacillus* group have apparently the same ability to degrade African locust bean proteins. Consequently, they could be used alternatively as starter in controlled fermentation to produce *afitin*, *iru* or *sonru*, taking into account their other technological performance and the physicochemical characteristics of each condiment. For example, *B. licheniformis* strain Y48H5 (BL1) and *B. subtilis* strain F24H10 (BS1) were found to have similarly high protease activities and to develop identical profiles when used for the fermentation of ALBP. But BS1 showed higher esterase as well as high protease activities. Both properties are required for the development of the texture and flavour of similar alkaline fermented condiments (Odunfa, 1985; Wand and Fung, 1996; Ouoba et al., 2003a, b). BS1 could be good choice to be used singly for controlled fermentation of African locust bean to produce good quality condiment. As mentioned, the degradation of ALBP varied significantly according to the strain and this is assumed to influence the nutritional status of *afitin, iru* or *sonru*. According to Fetuga et al. (1974) and Addy et al. (1995) and Ouoba et al. (2003a), the degradation of African locust bean is likely to produce essential amino acids and bioactive peptides useful for humans

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