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Optimal Conditions for Expression of Enzymatic Activities in *Bacillus* Strains Essential for Production of Soumbara, a Functional Fermented Food Involved in Blood Pressure Homeostasis

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Authors' contributions

This work was carried out in collaboration among all authors. C GTK and HGO designed the study. Authors HGO and YAK wrote the protocol validated by authors GTK YAK managed the literature search, performed the experimental and statistical analyses of the study and wrote the first draft of the manuscript. All authors read, corrected, managed and approved the final manuscript.

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ABSTRACT

Background: Soumbara is a traditional fermented food involved in the blood pressure homeostasis. The functional properties of this food strongly depend on the fermentative microbiota. In this study, we screened and identified at molecular level, some potential starters strain among the main microbiota associated with soumbara in Cote d'Ivoire, and investigated the conditions for optimal expression of their functional performance.
Methods: We screened and identified by ribosomal gene sequencing, interesting microbial strains and conditions for optimal expression of their functional performance notably proteolytic, lipolytic, pectinolytic, amylolytic and cellulolytic activities was investigated using semi quantitative method.
Results: The isolated microbiota was composed of 90.41 % of *Bacillus* strains, the rest being yeast

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and lactic acid bacteria. A total of 4 performant strains, specifically *Bacillus subtilis* (BS14P), *Bacillus subtilis* (KS16P), *Bacillus velezensis* (HS27M) and *Bacillus pumilis* (PS10P) were extracellular enzymes producing strains with halo diameter ranging between 1.9 and 2.8 cm. These strains grew optimally in the temperature range of 30- 35°C at pH 7-8. The largest enzyme producer *Bacillus velezensis* strain (HS27M) was remarkably able to grow well at relatively high temperature (40°C) and in a larger pH range (6-9). Proteolysis enzymes were produced optimally at 40-45°C, pH 7 whereas lipolysis occurred maximally at 40°C, pH 7. Likewise maximum pectinolytis, cellulolysis and amylolysis occurred at 45°C, pH range 7-8.

Conclusion: This study suggests *Bacillus velezensis* HS27M as valuable starter cultures for the production of soumbara with bioactive and organoleptic quality.

Keywords: Food microbiology; microorganisms; Bacillus; soumbara.

1. INTRODUCTION

Microorganisms are essential for the production of fermented food as their shape the overall quality of the products [1-5]. Many microbial species are known to be associated with soumbara, a traditional fermented food popularly consumed in Africa as condiment or food seasoning [6-8]. Soumbara is known as involved in the blood pressure homeostasis and strongly advised in-patient diet [9].

For production of this condiment, the African locust bean (*Parkia biglobosa*) must be fermented via a microbial activity mainly led by bacteria of the genus *Bacillus* notably *Bacillus subtilis* [10-13]. The metabolic activities include proteolysis [14-16], lipolysis [17] and degradation of poly and oligosaccharides [17-18].

These metabolic reactions lead to the formation of essential compounds such as monosaccharides, peptides, essential amino acids, ammonia, essential fatty acids, flavonoids, vitamin, and minerals [19-20,16-21,12-22] that shape the flavoring characteristics, the organoleptic properties and the bioactive quality of Soumbara. Additionally, certain compounds of antioxidant group including flavonoids, phenols present in Soumbara are believed to contribute to the regulation of blood pressure, against cardiovascular disease [23-25]. Hence, this fermented food is widely part of African diet particularly among populations suffering from high blood pressure [21-26].

However, this food is processed traditionally with no established safety and standard manutention procedures, resulting in variable quality of soumbara. Moreover, this food is liable to be associated with undesirable microorganisms susceptible to cause food poisoning [27-28,13], as the uncooked form is sometime directly seasoned on food, with increasing intoxication risk for consumers.

Previously, we reported for the first time the microbial diversity associated with soumbara from six main producing regions in Cote d’Ivoire. One of the main output of this study was the presence of non-fermentative and potentially pathogen microbiota consisting in *Bacillus cytotoxicus, Staphylococcus pasteuri, Staphylococcus sciuri*, *Streptococcus oralis, Micrococcus yunnanensis.* Together with many other studies, this emphasizes the need of standardizing the production process of soumbara, using valuable starter microbial culture to control the microbiota and ensure the quality of this condiment.

In this study, we screened and identified at molecular level, some potential starters strain among the main microbiota associated with soumbara in Cote d’Ivoire, and investigated the conditions for optimal expression of their functional performance.

2. MATERIALS AND METHODS

2.1 Sampling, Culture Conditions and Isolation of Microorganisms

Samples of soumbara were collected from six producing regions in north Côte d'Ivoire notably Poro (9°25′ N, 5°37′ W), Kabadougou (9°30′ 0″ N, 7°34′ 0″ W), Tchologo (9°35′ 0″ N, 5°11′ 0″ W), Bagoué (9°31′ 0″ N, 6°29′ 0″ W), Hambol (7°41′ 0″ N, 5° 1′ 0″ W), Béré (8°3′ 0″ N, 6°11′ 0″ W). In each region, a sample of 100 g was collected per zone and were mixed and then homogenized to finally obtain 1 global sample, each sample representing one region that was used for different microbiological analyzes [13]. Microorganisms were isolated using decimal

dilution method on different selective media notably YGC (for yeasts) (CONDA, Madrid, Spain), nutrient Agar for *Bacillus* (CONDA, Madrid, Spain), MRS for lactic acid bacteria, Baird Parker Agar for *Staphylococci* (CONDA, Madrid, Spain). The cultures were incubated at 30°C for 24-72 h depending on the microorganisms that were biochemically identified at first level and further identified using genomics methods.

2.2 Molecular Identification of Isolates

1 ml of an overnight pre-culture was transferred into an Eppendorff vial and centrifuged at 13,000 rpm for 10 min, then the pelleted cells were used for total DNA extraction according to cetyltrimethylammonium bromide (CTAB) test protocol [29]. Bacterial 16S rRNA genes were PCR-amplified with the universal primers FGPS 1509 (5' AAGGAGGGGATCCAGCCGCA 3') and FSGP 6 (5 'GGAGAGTTAGATCTTGGCTCAG 3') [30], whereas the amplification of fungal 5.8S rRNA gene was carried out using universal primers ITS1 (5’ TCCGTAGGTGAACCTGCGG 3’) and ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) [31]. PCR reactions were performed with a thermocycler (APPLIED BIOSYSTEM model 2720 THERMAL) in a final volume of 10 µL containing 1.5 ng/µL of DNA extract as template, 1X OneTaq Quick-Load 2X Master Mix (NEB, South Africa), 0.1 µM of each primer and 3.8 µL ultra-pure water. The program used to run the PCR reactions was set as follow. After an initial denaturation at 94°C for 5 minutes, reactions were run for 35 cycles, each cycle comprising: denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute 30 seconds. Finally, extension at 7 minutes at 72°C was carried out. PCR products were purified and then sequenced using the FGPS 1509 and FSGP 6 primer pair for bacterial strain and ITS1 primer for fungal strain. A bioinformatics analysis using the basic local alignment search tool (BLAST, blastN) from the NCBI database site (blast.ncbi.nlm.nih.gov/) was performed to identify the microbial strains.

2.3 Screening of Functional Microorganisms

Screening of enzymes producing strains as functional microorganisms was performed on nutrient agar at pH 7 ± 0.2. For this purpose, different substrates (citrus pectin, soluble starch, cellulose, skim milk, Tween 80 and olive oil) depending on the type of enzyme screened were added to the medium at 1 %. Five wells (0.3 cm diameter 2 mm deep) were made aseptically in the solid medium. Then, a 24 h microbial pre-culture was suspended in distilled sterile water to make a DO600 of 1. Then the wells were loaded with 10 µL of each microbial suspension (10^7 cfu/mL) and incubated for 48 h at 30°C. The clearance zones corresponding to pectinolytic, amylolytic, cellulolytic and lipolytic activity were detected by flooding the solid medium with iodine-potassium iodide solution. The detection of proteolytic activity was performed without flooding the medium as clear halo were directly visible.

2.4 Effect of Culture Conditions on Enzyme Production

Impact of culture conditions notably temperature and pH on the capacity of the microbial strains to produce extracellular enzymes was investigated on solid medium as described above. The cultures were incubated for 48 h at different temperatures (30, 35, 40, 45 and 50°C) and pH (5.0, 6.0, 7.0, 8.0 and 9.0). The pH of medium was adjusted with 1N HCl or 1N NaOH, to get different pH values. After incubation and revelation (described above), the halo diameter was measured as a function of the amount of enzymes yielded by the microorganism.

2.5 Effect of Environmental Conditions on Microbial Growth

The studied environmental conditions susceptible to impact the growth of strains analyzed were temperature, pH and oxygen availability. For analysis of temperature effect on the growth of screened microbial strains, one milliliter of microbial suspension prepared as described above was used to inoculate 25 ml of basic medium composed of 0.02% yeast extract; 0.28% (NH4) 2SO4; 0.6% K2HPO4; 0.2% KH2PO4; 0.01% MgSO4 at pH 6. The cultures were incubated at different temperatures (30, 35, 40, 45, and 50°C) for 48 h and the growth was monitored by reading the optical density (OD600) at 12 h, 24 h, 36 h and 48 h against the non-inoculated medium.

Effect of pH on microbial growth was investigated by using the basic medium at different pH ranging from 5 to 9, with incubation at 30°C. Impact of atmosphere conditions notably oxygen availability on microbial growth was analyzed by

incubating the cultures in aerobiosis and anaerobiosis at 30°C and pH 6. In aerobiosis conditions, cultures were maintained under shaking at 150 rpm whereas anaerobiosis conditions were achieved by covering the cultures with paraffin without shaking.

2.6 Statistical Analysis

All tests were performed in triplicate and the results were expressed as average ± standard deviation (SD). The significance of difference was tested using ANOVA (Analysis of variance) and Duncan Multiple Range Test (DMRT) at 95 % confidence under SPSS 15.0 package.

3. RESULTS

3.1 Different Species of Microbial Groups Isolated from Soumbara

Three bacterial groups essentially found in soumbara samples analyzed, were *Bacillus,* lactic acid bacteria and *Staphylococcus* Table 1. Additionally, another microbial group composed of fungi notably yeasts and molds, was also isolated Table 1. Regarding the abundance of microorganisms, *Bacillus* was the major microbial group isolated with 90.41 % of total isolates; this group included 10 identified species basing on the 16S gene sequencing. Only 6 yeasts and 7 lactic acid bacteria were isolated, corresponding to two and three species, respectively. The distribution of the isolated microbiota through the region is shown in the Table 1. From the microbiota isolated, hundred strains representing 1/3 of strains from each species were randomly chosen to be analyzed for functional properties.

3.2 Screening of Microorganisms with Functional Properties

From microorganisms, functional properties such as proteolytic, lipolytic, pectinolytic, amylolytic and cellulolytic activities are highly determining since they shape the overall quality of soumbara produced [17-23,12]. Hence, in this study, hundred isolated strains were analyzed to check their capability to express such functional properties and screen the performant strains. The results showed that, 51 strains were able to degrade and use pectin as carbon source with halo diameters ranging from 1 to 1.9 cm depending on the amount of enzyme produced. In addition, 57 strains were amylolytic enzyme producers with halo diameters between 1.2 and 2.4 cm; 56 strains were cellulolytic enzyme producers, having halo diameters in the range 1.4-2.8 cm; 55 strains able to degrade skim milk as proteolytic enzymes producers presented halo diameters between 2.1 and 2.7 cm and 42 lipolytic strains able to degrade olive oil, had halo diameters in the range 1.5-2.5 cm.

Of these 100 strains tested, those presenting enzymatic activities (74 strains), all were *Bacillus* strains. These *Bacillus* strains revealed to be multifunctional with the capability to produce all the enzymatic activities tested. In contrast, yeasts showed a poor enzyme production. Among the yeasts strains analyzed, only pectinolytic and amylolytic activities were hardly detectable in *Blastobotrys profilferans* (LO3) and *Meyerozyma caribbica* (S71) strains with halo diameter never exceeding 0.5 cm. On the other hand, lactic acid bacteria were not able to secrete any enzyme in the conditions of our study.

Accordingly, strains with interesting functional properties were screened among the *Bacillus* population. As the halo diameter is a function of the amount of enzyme produced [32-33], we considered as high enzyme producers, strains producing the largest diameter. Thus strain with a halo diameter between 1.9 and 2.8 cm were considered as high enzymes producers. On this basis, a total of 4 performant strains, specifically *Bacillus subtilis* (BS14P), *Bacillus subtilis* (KS16P), *Bacillus velezensis* (HS27M) and *Bacillus pumilis* (PS10P) were further studied Fig. 1. We next, investigated the impact of environmental conditions on the capacity of screened strains to efficiently produce these enzymes.

3.3 Microbial Growth Under Different Environmental Conditions

The growth of the performant *Bacillus* strains screened above, under various culture conditions such as temperature, pH and oxygen availability (atmosphere conditions), was studied.

The results indicate that all the bacterial strains analyzed, grow optimally in the temperature range of 30-35°C, reaching up to OD600 0.35 corresponding to 7.35 log (cfu/mL) Table 2. However, the strains *Bacillus velezensis* (HS27M) and *Bacillus pumilis* (PS10P) have a rapid growth capacity, with the maximum bacterial population reached within 24 h of culture as indicated by the OD600 Table 2

comparatively to the other strains notably *Bacillus subtilis* (BS14P) and *Bacillus subtilis* (KS16P) that reached their peak after 48 h. Additionally, the bacterial growth was also strong in *Bacillus velezensis* (HS27M) and *Bacillus pumilis* (PS10P) regarding the high peaks of OD600 obtained. Moreover, the strain *Bacillus velezensis* (HS27M) was particularly remarkable with a non-significant decreasing growth at 40°C, contrasting with the growth of the other strains.

Regarding the pH conditions, the *Bacillus subtilis* strains generally showed a favourable growth in pH 7-8 ranges, with the maximum at pH 8 Table 3). Out of this pH range, the growth of these strains is strongly impaired keeping less than 42 % of the maximum growth (data not shown). In *Bacillus pumilus* strain PS10P, the favorable pH range shifted to 6-7 with the maximum growth at pH 6 Table 3. Moreover, unlike the other strains, *Bacillus velezensis* (HS27M) was remarkably able to grow well in a larger pH range 6-9 with the maximum at pH 7. Additionally, the studied strains had as a common physiological trait, as they preferentially growth in aerobiosis conditions and almost failed to grow in anaerobiosis Table 4.

3.4 Microbial Growth in the Presence of Different Substrates

Analysis of microbial growth in the presence of different substrates showed that citrus pectin and skim milk were the best substrates as they promoted the growth of all the tested strains Table 5. Additionally, starch and cellulose also favored the growth of the different bacterial strains, but were poor substrates respectively for *Bacillus subtilis* strain KS16P and *Bacillus subtilis* strain BS14P. The lipidic substrates such as tween 80, repressed strongly the growth of bacteria analyzed allowing only 15 to 35 % of maximum cell density. However, this repression effect was less marked with olive oil another lipidic substrate, that hindered the growth of the *Bacillus subtilis* strain KS16P and *Bacillus subtilis* strain BS14P but in contrast promoted the growth of *Bacillus velezensis* (HS27M) and *Bacillus pumilis* (PS10P) Table 5.

3.5 Influence of pH and Temperature on Enzymes Production

Enzyme production was essayed using semi-quantitative approach based on halo diameter as visible and measurable indicator. In this approach, the standard inoculum size (10 μL of bacterial suspension DO600= 1) used for each tested strain corresponded to 3.105 cells. This allowed using approximately the same inoculum size for all strains studied. In these conditions, as halo diameter depends on the quantity of enzyme produced [34-35], it was possible to evaluated enzyme production based on halo diameter.

From the results, it was observed that the culture temperature had a great influence on enzymes production in the bacterial strains analyzed. As a general trend, a larger halo diameter for pectinolytic, amylolytic and cellulolytic enzymes was obtained at 30 and 35°C with *Bacillus subtilis* (BS14P), *Bacillus subtilis* (KS16P) and *Bacillus pumilis* (PS10P). However, in these three strains, the maximum proteolytic enzyme production occurred at 40 or 45°C depending on the strain. Lipolytic enzymes production was the most susceptible to temperature change since strains behaved differently. Hence, *Bacillus subtilis* (BS14P) presented maximum production at 30°C while *Bacillus pumilis* (PS10P) and *Bacillus subtilis* (KS16P) produced maximum lipolytic enzymes at 35 and 40°C, respectively Fig. 2. In contrast, the temperature influenced differently enzyme production in *Bacillus velezensis* (HS27M). In this particular strain, the halo diameter of yielded enzymes increased progressively with increasing temperatures to reach the maximum diameter at 45°C. Moreover, *Bacillus velezensis* (HS27M) was in general the largest enzymes producer among the strains studied with halo diameter reaching more than 2.5 cm for all targeted enzymes Fig. 2.

The pH also influenced enzyme production during bacterial growth. Maximum halo diameter was obtained in pH range 7-8 for pectinolytic, cellulolytic and amylolytic enzymes while proteolytic and lipolytic enzymes were produced in a narrow range of pH around 7 Fig. 3.

4. DISCUSSION

In the present study, the distribution of isolated microbiota through the regions revealed a wide diversity of microorganisms associated with soumbara in Cote d'Ivoire, *Bacillus subtilis* being the major species whatever the studied region. Likewise, previous studies on the microbiology of the fermentation of African locust bean seeds have identified bacteria of the genus *Bacillus*, predominantly *Bacillus subtilis* as the main microorganims responsible for fermentation [11-36,13-37].

Table 1. Microorganism isolation of soumbara from the different producing region

Microbial groups	Number of strains						Total strains isolated	Identified species
	Béré	**Poro**	**Kabadougou**	**Tchologo**	**Bagoué**	**Hambol**		
Bacillus	38	45	35	47	49	41	255	- *Bacillus subtilis* - *Bacillus velezensis* - *Lysinibacillus macroide* - *Bacillus pumilis* - *Bacillus endophyticus* - *Bacillus oleronius* - *Bacillus drentensis* - *Paenibacillus xylanilyticus* - *Rummelliibacillus stabekissii* - *Bacillus cytotoxicus*
Yeasts	0	0	3	1	2	0	6	- *Meyerozyma caribbica* - *Blastobotrys profilferans*
Lactic acid bacteria	2	0	1	2	0	2	7	- *Streptococcus oralis* - *Enterococcus faecium* - *Weissella cibaria*
Other group	0	1	1	0	1	0	3	- *Micrococcus yunnanensis* - *Acinetobacter variabilis* - *Paracoccus siganidrum*
Staphylococci	0	0	2	0	1	1	4	- *Staphylococcus pasteuri* - *Staphylococcus sciuri*
Mold	0	1	2	0	4	0	7	- *Penicillium sclerotiorum* - *Penicillium chrysogenum* - *Absidia corymbifera* - *Cladosporium uredinicola* - *Fusarium oxysporum* - *Aspergillus flavus*

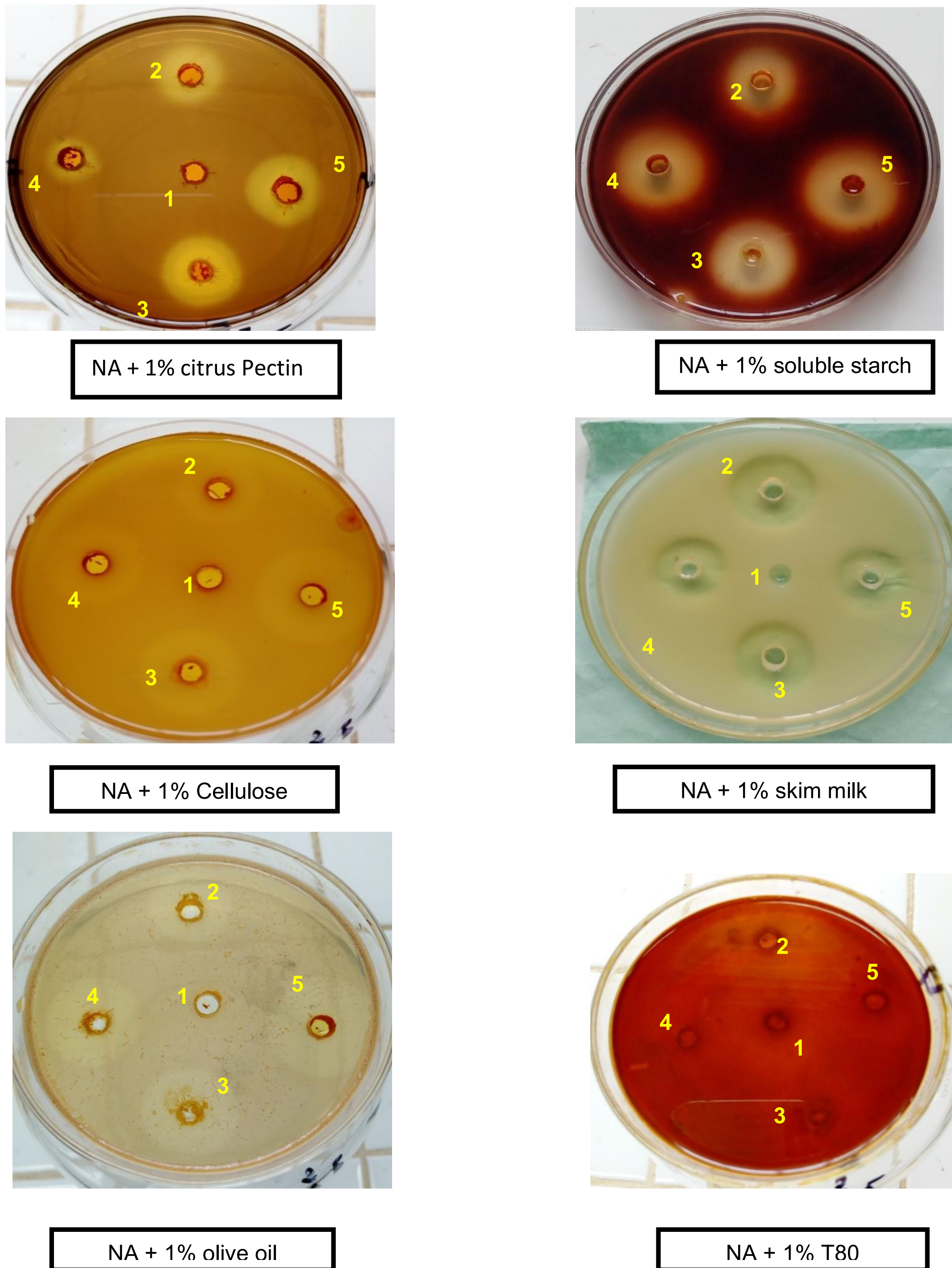

Fig. 1. Screening of *Bacillus* producers of enzymes on solid medium

The fives wells were seeded as follow, 1: Without strain; 2: Bacillus subtilis (BS14P); 3: Bacillus velezensis (HS27M); 4: Bacillus subtilis (KS16P); 5: Bacillus pumilis (PS10P). Enzymatic activity is detected by clear zones around the colonies using NA: Nutrient Agar as basic medium. Substrates are citrus pectin for pectinolytic activity, soluble starch for amylolytic activity, cellulose for cellulolytic activity, skim milk for proteolytic activity and olive oil for lipolytic activity

Table 2. Effect of temperature on microbial growth

	Optimal OD (nm)				
	30°C	**35°C**	**40°C**	**45°C**	**50°C**
B. subtilis (BS14P)	0,164±0,001d (48 h)	0,127±0,001d (48 h)	0,06±0,01d (12h)	0,048±0,001c (24h)	0,045±0,001c (12h)
B. velezensis (HS27M)	0,353±0,001a (24h)	0,35±0,001a (24h)	0,331±0,001a (12h)	0,09±0,01b (12 h)	0,114±0,001a (24h)
B. subtilis (KS16P)	0,224±0,001c (48h)	0,19±0,001c (48h)	0,13±0,01c (48h)	0,092±0,001b (24h)	0,011±0,001d (36h)
B. pumilis (PS10P)	0,35±0,001a (24h)	0,277±0,001b (24h)	0,19±0,001b (12h)	0,189±0,001a (36h)	0,052±0,001b (36h)

Each value was performed in triplicate and the results were expressed as average ± standard deviation
Different letters (a, b, c...) on the same column indicate a statistical difference (p ≤ .05)

.Table 3. Effect of pH on microbial growth

	Optimal OD (nm)				
	pH5	**pH6**	**pH7**	**pH8**	**pH9**
***B. subtilis* (BS14P)**	0,001±0,001b (12h)	0,097±0,001c (48h)	0,164±0,001d (48h)	0,228±0,001b (24h)	0,042±0,001b (48h)
***B. velezensis* (HS27M)**	0,024±0,001a (12h)	0,25±0,002b (12h)	0,353±0,002a (24h)	0,268±0,001a (24h)	0,267±0,001a (36h)
***B. subtilis* (KS16P)**	0,001±0,001b (12h)	0,089±0,001d (12h)	0,224±0,001c (48h)	0,103±0,001d (36h)	0,04±0,01b (12h)
***B. pumilis* (PS10P)**	0,001±0,001b (12h)	0,35±0,001a (24h)	0,298±0b (48h)	0,191±0,001c (24h)	0,044±0,001b (24h)

Each value was performed in triplicate and the results were expressed as average ± standard deviation
Different letters (a, b, c...) on the same column indicate a statistical difference (p ≤ .05)

Table 4. Effect of oxygen availability on microbial growth

	aérobiose			anaérobiose		
	Initial OD (nm)	**Optimal OD (nm)**	**Final OD (nm)**	**Initial OD (nm)**	**Optimal OD (nm)**	**Final OD (nm)**
***B. subtilis* (BS14P)**	0,006±0,001c (0h)	0,164±0,001d (48 h)	0,164±0,001d (48 h)	0,006±0,001c (0h)	0,038±0,001c (36h)	0,026±0,001b (48h)
***B. velezensis* (HS27M)**	0,071±0,002a (0h)	0,353±0,001a (24h)	0,322±0,001a (48h)	0,071±0,002a (0h)	0,083±0,001b (36h)	0,083±0,001a (48h)
***B. subtilis* (KS16P)**	0,002±0,001c (0h)	0,224±0,001c (48h)	0,224±0,001c (48h)	0,002±0,001c (0h)	0,026±0,001d (24h)	0,012±0,001c (48h)
***B. pumilis* (PS10P)**	0,026±0,001b (0h)	0,35±0,001a (24h)	0,298±0,001b (48h)	0,026±0,001b (0h)	0,113±0,001a (12h)	0,081±0,001a (48h)

Each value was performed in triplicate and the results were expressed as average ± standard deviation
Different letters (a, b, c...) on the same column indicate a statistical difference (p ≤ .05)

The screening of isolated microbiota showed an interesting multifunctional trait of *Bacillus* strains with the capability to produce all the enzymatic activities tested such as pectinolytic, amylolytic, proteolytic, cellulolytic and lipolytic activities. This result corroborate that of Aderibigbe et al. 1990 [38] who reported that *Bacillus* from Soumbara produce a diversity of extracellular hydrolases. The strong capacity of *Bacillus* strains to produce various extracellular enzymes makes them one of the most used bacteria in food industry for fermented foods production and also used as additives in animal feed [39-40]. Our study shows that these bacteria could also be the most suitable candidates for improving fermentation of African locust bean seeds in order to have a good quality soumbara.

Table 5. Microbial growth in the presence of different substrates

	Bacillus subtilis **(BS14P)**	***Bacillus velezensis*** **(HS27M)**	***Bacillus subtilis*** **(KS16P)**	***Bacillus pumilis*** **(PS10P)**
BM	0,164±0,001d (48h)	0,353±0,001d (24h)	0,224±0,001d (48h)	0,35±0,001e (24h)
BM+Pectin	0,489±0,001a (24h)	0,655±0,001a (48h)	0,295±0,001b (24h)	0,591±0,001b (48h)
BM+ Starch	0,305±0,001b (48h)	0,315±0,001f (48h)	0,101±0,001g (12h)	0,657±0,001a (36h)
BM+Cellulose	0,067±0,001f (12h)	0,398±0,001c (48h)	0,308±0,001a (48h)	0,239±0,001f (48h)
BM+Smik milk	0,252±0,001c (48h)	0,333±0,001e (24h)	0,274±0,001c (48h)	0,498±0,001c (24h)
BM+Olive oil	0,032±0,001g (36h)	0,417±0,001b (36h)	0,115±0,001f (12h)	0,388±0,001d (36h)
BM+Tween 80	0,082±0,001e (36h)	0,151±0,001g (36h)	0,135±0,001e (36h)	0,043±0,001g (48h)

BM: Basic Media

Each value was performed in triplicate and the results were expressed as average ± standard deviation. Different letters (a, b, c...) on the same column indicate a statistical difference (p ≤ .05)

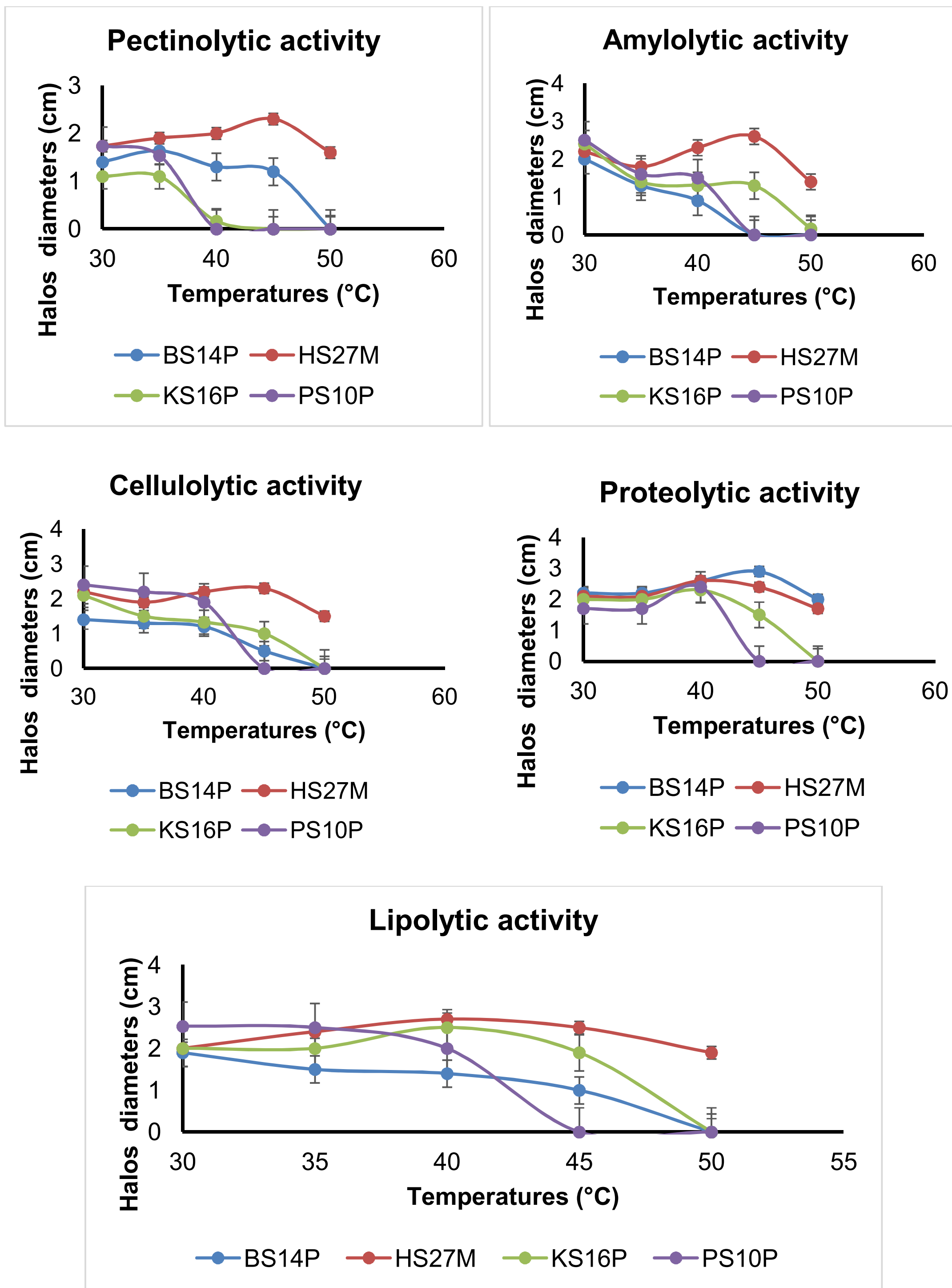

Fig. 2. Influence of temperature on enzyme production

Basic culture medium is NA ; Substrates are citrus pectin for pectinolytic activity, soluble starch for amylolytic activity, cellulose for cellulolytic activity, skim milk for proteolytic activity and olive oil for lipolytic activity ; Cultures were incubated for 48 hrs at different temperatures

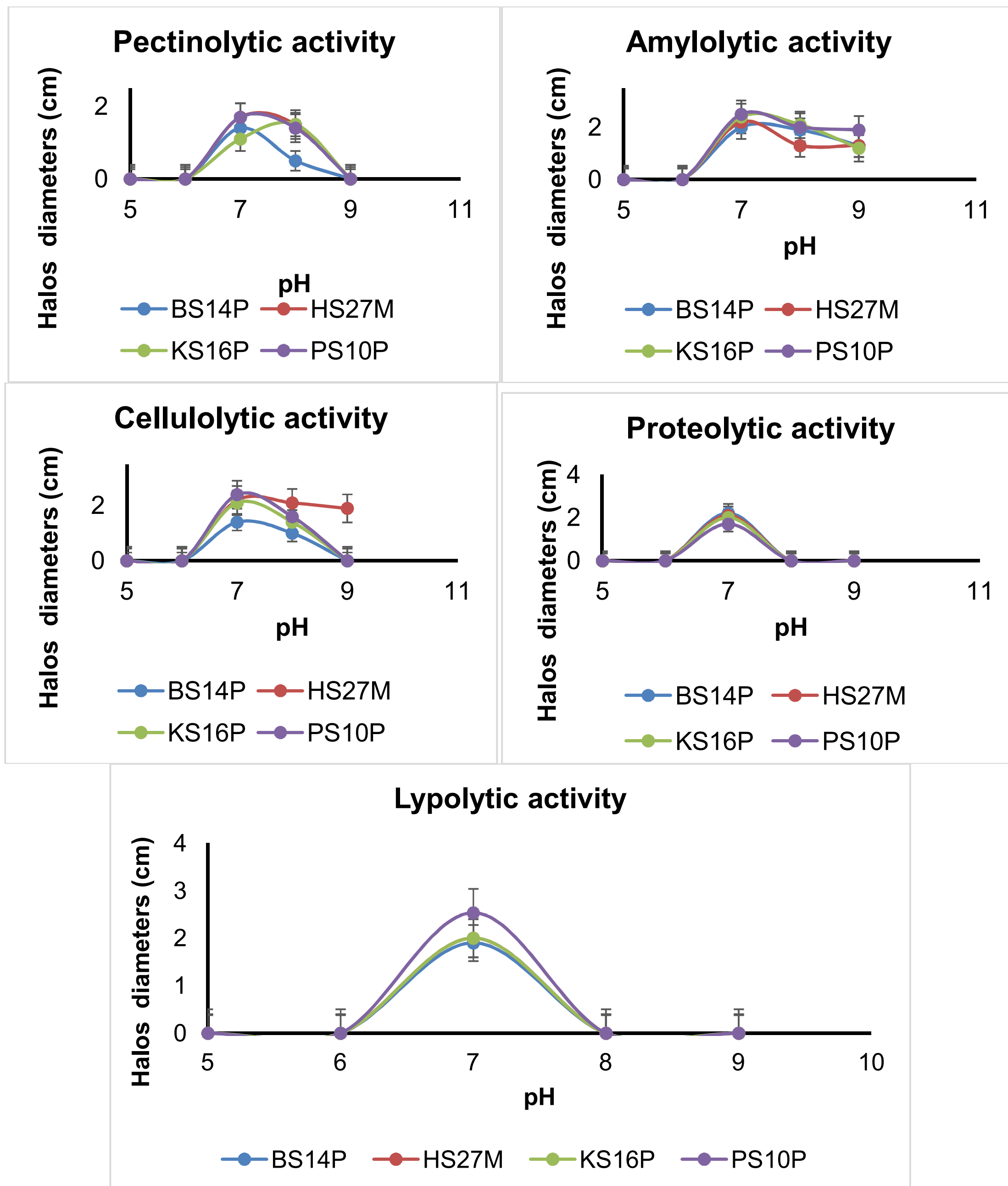

Fig. 3. Influence of pH on enzyme production

Basic culture medium is NA ; Substrates are citrus pectin for pectinolytic activity, soluble starch for amylolytic activity, cellulose for cellulolytic activity, skim milk for proteolytic activity and olive oil for lipolytic activity ; Cultures were incubated for 48 hrs at different pH

From the microbiota isolated, we have screened precisely two potential starter strains *Bacillus subtilis* KS16P and *Bacillus velezensis* (HS27M). Unlike *Bacillus subtilis* that was widely reported in Soumbara fermentation, the species *Bacillus velezensis* was not reported before in the fermentation of African locust bean seeds from any other country. Regarding the growth conditions, the *Bacillus velezensis* strain behaved differently comparatively to the other *Bacillus* strains. In fact the *Bacillus velezensis* was able to grow well at relatively higher

temperature (40°C) and in a larger pH range (6-9), what has been previously reported by Ruiz-Garcia et al. 2005 [41]; while the growth of other *Bacillus* strain stayed at 35°C and in a narrow pH range (7-8). Previous studies have also showed that, during the fermentation of soumbara, the increase in temperature (from 26 to 46°C) corresponds to an increase in the microbial population and in pH (from 7.1 to 7.9) [42] and the final pH is more generally around 7 [43]. However, the soumbara sample we had collected in this study had a final pH around 8.7; this could strongly be linked to the particular presence of the bacterial strain *Bacillus velezensis* that grew well *in vitro* at pH 9. The high alkaline pH of our sample may be due to the release of alkaline substrates such as amino acids, ammonia from microbial activity during the fermentation process, which caused the pH of the fermenting material to increase up to 8. Several studies have also suggested that, the final pH of soumbara may be essentially assigned to microbial activity during fermentation [44-47].

Analysis of functional properties showed that enzyme production in isolated bacteria depends on environmental conditions notably pH and temperature. Proteolysis enzymes were produced optimally at 40 or 45 °C, pH 7 depending on the strain whereas lipolysis occurred maximally at 40 °C, pH 7. Likewise maximum pectinolytic, cellulolysis and amylolysis occurred at 45 °C, pH range 7-8. Previous reports indicated varying optimal temperature for the production of protease by *Bacillus subtilis* [48]. These authors observed maximum yield of proteins at 40 °C at the third day of fermentation of African locust bean seed. Moreover, published research on biochemical changes during fermentation of African locust bean seed has shown that proteolysis is the main activity leading to the release of peptides, ammonia and amino acids such as glutamic and aspartic acid knowned as flavor enhancers [17-21,12]. According to N'Dir et al., 1994 [42], the flavor and taste of soumbara would be the positive consequence of the proteolytic activity of microorganisms and the appearance of ammoniacal compounds, while lipolytic activity resulting from the hydrolysis of triglycerides or more complex phospholipids contained in fatty acids strongly impacts the physico-chemical and sensory characteristics of soumbara [12-49]. Microbial activity also involves the excretion of high texturizing exopolysaccharides and lipopeptides [6]. According to N'Dir et al. [50], the preservation of soumbara is, among other things, linked to the production of antifungal lipopeptides (iturin A and surfactin) by various strains of bacilli isolated after the fermentation process. In addition, the microbiota produces certain hydrolases, such as amylase, galactanase, galactosidase, and 1-6 fructoranosidase, which are involved in the degradation of carbohydrates into more digestible sugars [51-38,18].

In this study, the observed variability of pH and temperature conditions for these enzymatic properties to occurs, indicates the importance of adaptation for the microbiota to operate efficiently by expressing their different potential in changing conditions. In this context, microbial strains *Bacillus velezensis* (HS27M) was shown to be the most capable to induce or to resist to the change in the fermentation conditions. Furthermore, this strain was the highest enzymes producer among the strains studied with halo diameter reaching more than 2.5 cm at 45 °C for all targeted enzymes. The strains *Bacillus velezensis* (HS27M), seems to be the most well adapted strain to the African locust bean fermentation in Cote d'Ivoire.

5. CONCLUSION

This study elucidated the exact role of the *Bacillus velezensis* strains and evidenced their adaptation in the fermentation of African locust bean seeds, regarding the interesting techno-functional performances notably the strong enzymatic activities expressed *in vitro* and the predominance of this species in the final product (soumbara). *Bacillus velezensis* strain HS27M and *Bacillus subtilis* strain KS16P are probably the initiators of the fermentation of African locust bean seeds and the key players in the fermenting matter. These strains could be used as valuable starter to improve the sensory properties of foods, which include organoleptic characteristics (appearance, aroma and flavor) of soumbara.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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