The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology (2017), 41(1), 131-146

ORIGINAL RESEARCH PAPER

SELECTION OF POTENTIAL *Bacillus* STARTERS FOR COCOA BEANS FERMENTATION IMPROVEMENT

WILFRIED YAO[1], GINETTE DOUE[1*], HONORÉ OUATTARA[1], BERNADETTE GOUALIE[1], GISÈLE KOUA[1], SÉBASTIEN NIAMKE[1]

[1]Laboratoire de Biotechnologies, UFR Biosciences, Université Félix Houphouët-Boigny, 22 BP 582 Abidjan 22, Côte d'Ivoire
Corresponding author: gladysdoue@yahoo.com

Received on 10th November 2016
Revised on 6th June 2017

Bacillus sp. strains isolated from six main Ivorian cocoa producer regions were investigated based on their biochemical properties in order to select the best one as potential starter. Three mains technological and useful properties for good cocoa beans fermentation were monitored for 970 isolated *Bacillus sp*. strains. Thus, 44.54% pectinolytic *Bacillus sp*. strains revealed by clear halo diameter in plate assay were confirmed by enzymes production (pectinase and polygalacturonase) in liquid medium. Moreover, 51.10% of these strains displayed both the ability to acidify the medium and to degrade the citrate. Among these pectinolytic enzymes producing strains previously selected, ten were able to maintain this activity in conditions of varying pH and temperature as same as fermentation heap with an activity ranged within 0.20±0.02-0.51±0.05µmol/min/mg of proteins for polygalacturonase (PG) and 0.22±0.04-0.76±0.06µmol/min/mg of proteins for pectinase. Four of them (T_9G_4, $T_{11}I_{10}$, T_3D_{23} and T_4A_{44}) selected as the best based on pectinase production level were identified as *Bacillus thuringiensis.* These strains were able to continuously produce both pectinolytic enzymes in different conditions of organic components: acids (acetic and lactic) influence with 0.2% as limit of tolerance, citric acid influence (up to 1% of tolerance) and ethanol influence higher than 8%. The technological properties of the four best strains highlighted in this study may make them suitable candidates as starter for cocoa fermentation control.

Keywords*: Bacillus*, technological properties, starter, Ivorian cocoa, fermentation

Introduction

Bacillus sp., gram-positive bacteria, are part of the major groups of microorganisms involved in cocoa fermentation besides yeast, acetic acid bacteria (AAB) and lactic acid bacteria (LAB). Cocoa beans fermentation, which is the first

step in the chocolate-making chain, is one of the key steps for ensuring the marketable quality of cocoa beans and chocolate (Pereira et al., 2013; Samagaci et al., 2014). However, this microbial process remains difficult to control leading to inconsistent quality cocoa, especially since these microorganisms are from the hazardous contamination of beans by indigenous strains of cocoa pods and local fermentative practices (Schwan and Wheals, 2004). This variability of cocoa beans quality is recurrent and causing significant economic losses for producers (Camu et al., 2007). In this context, many studies have suggested the use of microbial starter culture as the best approach to improve fermentation process (Passos et al., 1984; Hansen et al., 1998; Hashim et al., 1998; Nielsen et al., 2005; Papalexandratou et al., 2011).

For the best of our knowledge, none *Bacillus sp*. has been used in microbial cocktail, as starter in any assay aiming to control and standardize the cocoa fermentation as no valuable role have been reported for them. Contrary to this, they are thought to be responsible for off flavours of fermented cocoa beans. (Schwan and Wheals, 2004). However, an interest seems to emerge about these bacteria through a number of studies aiming to clarify their role in this process. Thus, the results achieved by Schwan (1998) clearly showed that chocolate obtained with a high presence of *Bacillus* gave less off flavour compared with the chocolate obtained without *Bacillus* strains. More recently, Ouattara et al. (2008) reported their implication in the production of pectinolytic enzymes and assumed that these bacterial strains may act as complementary partner to yeast strains in the depectinization of pulp during cocoa fermentation (Ouattara et al., 2011). Furthermore, several studies have showed that *Bacillus* species are present throughout the spontaneous and natural cocoa fermentation at higher level than yeasts, LAB and AAB (Rooijackers et al., 2012). In addition, they are, as same as AAB and LAB, responsible for acidification and pH increase of the fermentation heaps (Kouame et al., 2015a; Kouame et al., 2015b).

The control of this process requires a well understanding of cocoa microbiota physiology and screening of strains with high biochemical performances. A deep understanding of *Bacillus* role in the cocoa fermentation, notably their technological properties such as pectinolytic enzymes production, acidification capacity and citrate metabolism, is indispensable for their use as starter in microbial cocktails for control of the fermentation process. The aim of this work is to highlight these technological properties for *Bacillus* strains involved in Ivorian cocoa fermentation in order to select the best one as potential starter.

Materials and methods

Fermentation conditions

The cocoa pods were harvested at farms from six mains Ivorian cocoa producing regions: Agnéby-Tiassa (5°59’ North 4°28’West), Guemon (6°29'41" North

6°57'59" West), Indenié-Djuablin (7° 7' 52" North 3° 12' 15" West), Lôh-Djboua (5°55' North 5°37'West), Nawa (9°39'0" North 5°1'60" West) and Sud-Comoé (5°28'06' North 3°12'25' West).

The spontaneous cocoa beans fermentation was performed in National Flowers Center of Félix HOUPHOUËT-BOIGNY University (temperature around 28-30°C and humidity 60-65%), in traditional conditions by heap fermentation during 6 days. The fermenting mass (100kg) set on banana leaves and covered with banana leaves were constituted of mixed genotypes (Forastero, Trinitario and Criollo cultivars). The fermenting heap was mixed and 200g beans were collected in sterile Stomacher bag for microbial analysis each 12h of fermentation. The pH and temperature were also recorded at the same depth on the fermenting heap, with pH-meter and thermometer.

Isolation of bacterial strains

Isolation was performed according to the standard method described by Nielsen et al. (2007). An amount of 25g of fermenting cocoa beans was homogenized in 225mL sterile peptone water (pH 7.0). After appropriate dilution of samples in sterile saline solution, 0.1 mL from each dilution was inoculated onto duplicate plates of nutrient agar (Scharlau Microbiology, Spain) supplemented with 0.1% nystatin to inhibit the fungal growth. The culture was incubated at 30°C for 48h. *Bacillus sp*. isolates, identified using conventional colonial morphology, Gram staining and biochemical reactions according to Bergey's manual of systems bacteriology, were stored at -80ºC in nutrient broth supplemented with glycerol 20% in Eppendorf tubes, for further studies.

Screening of Bacillus strains for pectinolytic enzymes production

Pectinolytic enzymes production in Solid Medium

Pectinolytic strains were screened using the method described by Soares et al. (1999) and modified by Ouattara et al. (2008). Basal mineral medium was prepared with 0.28% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.01% $MgSO_4$, 0.2% KH_2PO_4, 0.02% yeast extract, 1% pectin for pectinase or 1% polygalacturonic acid for polygalacturonase (PG) and 1.7% agar, pH 6.8. Four wells of 0.5cm in diameter and 2 to 3mm in depth were aseptically made in the medium. Then, pure *Bacillus* culture was suspended in saline tryptone to have an optical density of 1 at 600nm (corresponding to a cell concentration of 10^7 cfu/mL). 7µL of the suspension were subsequently used to load the wells. All the wells of the same plate were inoculated with a single suspension and incubated at 30ºC for 48h.

After incubation, the solid culture medium was flooded with a lugol solution 1.83% (0.3g iodine + 1.52g potassium iodide in 100mL of distilled water) to reveal clear zones around the wells, indicating pectinolytic production and halo diameter was measured.

Pectinolytic enzymes production in liquid medium

The ability of strains selected in solid medium to produce pectinolytic enzymes were confirmed and evaluated in liquid medium.

Culture conditions and enzymes production

Liquid mineral medium was prepared with 0.28% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.01% $MgSO_4$, 0.2% KH_2PO_4, 0.02% yeast extract, 1% pectin for pectinase or polygalacturonic acid for polygalacturonase (PG), and pH 6.8. 1mL of bacterial suspension prepared as described above was used to inoculate this medium. Cultures were grown in 50mL Erlenmeyer flasks with 10mL of medium in a rotary shaker (150rpm) at 30°C for 48h, in aerobic conditions. Biomass was separated by centrifugation at 15000rpm for 30min at 4°C. Enzymes activity was measured in the cell-free supernatant.

Protein and enzyme assays

The pectinase and polygalacturonase (PG) activities were determined using pectin and polygalactouronic acid as substrates (Minjares-Cassanco et al., 1997). The reaction mixture containing 1% substrate prepared in phosphate buffer 0.1M pH 6 and 100µL of cell-free supernatant were incubated at 40ºC for 30min. After incubation, 250µL of DNS solution were added to stop the reaction and tubes were kept in boiling water for 5 minutes. The absorbance was read at 575nm after addition of 2 mL of distilled water using UV-visible spectrophotometer (Pioway Medical Lab - UV752). The amount of released reducing sugar was quantified using galacturonic acid as standard.

The enzymes activity was defined as the amount of enzyme required to release one micromole equivalent of galacturonic acid per minute under assay conditions.

Proteins content was assessed by Lowry *et al.* (1951) method using bovine serum albumin as standard.

Analysis of pectinolytic Bacillus sp. isolates for additional properties

Acidification of the fermenting cocoa beans is one of the most important factors indispensable for good quality cocoa (Afoakwa et al., 2013). Pectinolytic *Bacillus* strains identified among the isolates were subjected to analysis for their ability to acidify the medium according to the method previously described by Aydin and Aksoy (2009) with slight modification. Acid production was assessed by formation of yellow area in the tube with or without gas production.

Pectinolytic *Bacillus* strains previously identified by their ability to acidify the medium were tested for their ability to degrade citrate by the method described by Beishir (1991) on Simmons citrate medium. The medium was inoculated with pure pre-culture and incubated at 30°C for 48h. After incubation, the blue colonies were those able to metabolize citrate.

16S rRNA gene PCR and sequence analysis

The isolates of *Bacillus* were grown on Luria Bertani (LB) agar medium during 24h of incubation at 30°C.

A loopful of pure culture was suspended in 100µL of sterile distilled water and this sample was used for colony PCR. In this technique, the medium was constituted by microorganisms from one pure colony. After the first stages of denaturation, the released DNA was used as matrix for amplification.

PCR amplification of ribosomal 16S gene

The hypervariable regions (V_1, V_2 and V_6) of the different *Bacillus sp.* strains were amplified by using universal primers F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R520 (5'-ACCGCGGCTGCTGGC-3') (Ouattara et al., 2011) for colony PCR.

Each mixture (final volume 50µL) contained about 1µL of sample 1.25U of Taq DNA polymerase (Biolabs, Lyon France), 5µL of 10x Taq Buffer (10mM Tris-HCl, 50mM KCl, 1,5mM $MgCl_2$), 1µL of 10mM dNTP, 2µL of each primer (10mM) (Eurofins, Genomics, Germany) supplemented by sterile water Milli-Q.

PCR amplification was carried out in a thermocycler (Sensoquest Labcycler). After an initial denaturation at 95°C for 4min, reactions were run for 35 cycles, each cycle comprising denaturation at 95°C for 1min, annealing at 56°C for 1min, extension at 72°C for 1min; and final extension at 72°C for 10min.

Gel electrophoresis

After migration of the products realised at 70 V for 2h in a tank, amplicons were revealed by incubating them on 0.8% agarose gel electrophoresis in a 1x phosphate TAE buffer containing bromide of ethidium. The molecular weight of the products was estimated in reference to a molecular weight marker (Biolabs, France) of 500bp.

Sequencing and analysis of hypervariable sequence of 16S RNA gene amplified

The PCR products were purified using the nucleospin^R Gel and PCR Clean-up kit (MachereyeNagel, Germany) and were then sequenced by MWG Eurofins using the primer F27. The gene sequences obtained were compared to the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Effect of environmental conditions on pectinolytic enzymes production

The ability of the selected strains to withstand the changes in fermentation conditions was evaluated at this level.

Temperature influence

The influence of temperature on pectinolytic enzymes produced by *Bacillus* strains was evaluated. The modulation of enzymes production by temperature was studied on liquid medium described above. Cultures were incubated for 48h at different temperatures (30, 35, 40, 45 and 50ºC) and then assayed for pectinase and polygalacturonase (PG) activity.

Influence of pH

The influence of the pH on pectinolytic enzymes produced by *Bacillus* strains was analysed. The modulation of enzymes production by pH was studied on liquid

medium previously described. The basal medium was adjusted at different pH (4, 5, 6 and 7). All cultures were incubated during for 48h at 30°C and then assayed for pectinase and polygalacturonase (PG) activity.

Influence of ethanol and organic acids on pectinolytic enzymes production by Bacillus sp. strains

In addition to their ability to withstand the changes in temperature and pH of the fermentative heap, the ability of the selected strains to maintain their pectinolytic enzymes production despite different stress of the fermentative medium was evaluated at this level.

Effect of ethanol

To evaluate the effect of ethanol concentration on pectinolytic enzymes production, different concentrations of ethanol (0, 2, 4, 6 and 8%) were added to the basal mineral medium. After incubation, pectinase and polygalacturonase (PG) were assayed as previously described.

Effect of organic acids

The effect organic acids (citric, lactic and acetic acids) on pectinolytic enzymes production was evaluated as follow: different compounds were added to the basal mineral medium at concentrations 0.2 to 1% for citric acid, 0.1 to 0.6% for lactic acid and 0.05 to 0.5% for acetic acid. After incubation, pectinase and polygalacturonase (PG) were assayed as previously described.

Results and discussion

Highlighting Bacillus strains technological activity

Overall, 970 Bacillus strains associated with cocoa beans fermentation were isolated from six Ivorian regions and analysed for three main technological properties (pectinolytic enzymes production in solid and liquid medium, acidification capacity and citrate degradation). These results showed that the isolated strains have an unequal distribution according to the studied regions with Agnéby-Tiassa recording the highest (32.34%) number of isolates (Table 1).

This variability is not surprising since the local geographic area is an important factor influencing the composition of the microflora involved in cocoa fermentation as reported by Schwan and Wheals. (2004). However, to identify potential starters among these isolates, it appears important to screen them based on their ability to display different types of metabolic properties susceptible to play a key role in the fermentative process (Afoakwa et al., 2013). Pectinolytic enzyme production, acidification capacity and citrate metabolism are part of these important properties. They are important and desirable properties for adequate cocoa fermentation (Thompson et al., 2001; Aydin and Aksoy, 2009; Afokwa et al., 2013) and strains exhibiting them are potentially interesting as starter.

Table 1. Distribution of *Bacillus sp.* strains isolates according to their technological properties per regions

Regions	S. I.	P. S. I.	P. S. A. C.	P. S. A. C. D.
Lôh-Djboua	173 (17.83%)	78 (18.05%)	57 (17.87%)	40 (24.54%)
Sud-Comoé	113 (11.64%)	73 (16.90%)	66 (20.69%)	39 (23.93%)
Agnéby-Tiassa	314 (32.34%)	104 (24.08%)	67 (21.00%)	55 (33.74%)
Guemon	122 (12.57%)	41 (9.49%)	32 (10.03%)	11 (6.75%)
Nawa	121 (12.47%)	55 (12.73%)	27 (8.46%)	07 (4.29%)
Indenié-Djuablin	127(13.08%)	81 (18.75%)	70 (21.95%)	11 (6.75%)
Total isolement	**970**	**432 (44.54 %)**	**319**	**163 (51.10%)**

Note: With regard to the technological properties, they have been studied by steps. The strains which responded positively to the first test were selected for the second test. S. I.: Strains Isolated; P. S. I.: Pectinolytic Strains Isolated; P. S. A. C.: Pectinolytic Strains with Acidifying Capacity; P. S. A. C. D.: Pectinolytic Acidifying Strains with Citrate Degradation.

Pectinolytic enzymes production in both solid and liquid mediums

Pectinolytic Activity of Bacillus Strains

Among the 970 isolated strains, 44.54% exhibited pectinolytic activity in plate assay (solid medium) as revealed by the clear halo and confirmed in liquid medium. (Table 1). These isolates were spread differently from one region to another with values ranging within 9.49% and 24.08%. Among these regions, Agnéby-Tiassa recorded the highest number of pectinolytic strains isolated (24.08%), while Guemon exhibited the smallest number (9.49%) (Table 1). Previous study reported that more than 90% of *Bacillus* strains isolated from Ivoirian cocoa fermentation were pectinolytic strains (Ouattara et al., 2008). These results clearly highlight the role of *Bacillus* strains in the degradation of pectin as yeasts (Schwan et al., 1997). Furthermore, these isolates of *Bacillus sp*. strains may play a predominant and active part in this process as their proportion is higher than that (17%) reported for the yeasts by Samagaci et al. (2014).

Nature of pectinolytic enzymes produced

Bacillus sp. strains previously identified as pectinolytic strains were able to produce two types of pectinolytic enzymes: pectinase and polygalacturonase (PG) in both mediums (Table 2). Indeed, these pectinolytic *Bacillus sp.* strains showed in solid medium halo diameter between 1 and 3.4cm for pectinase and between 1 and 3.2cm for polygalacturonase (PG). These results were confirmed in liquid medium where these *Bacillus sp*. strains were able to maintain their pectinolytic enzymes activities. The table 2 showed that these strains produced both types of pectinolytic enzymes such as pectinase and polygalacturonase (PG) with different

activity level ranged within 0.13±0-1.26±0.01µmol/min/mg of proteins and 0.09±0-0.75±0.01µmol/min/mg of proteins, respectively.

Previous study reported that *Bacillus sp.* strains isolated from Ivoirian cocoa fermentation mainly produced both types of pectinolytic enzymes, polygalacturonase (PG) and pectinase (Ouattara et al., 2008), unlike yeasts that produce only polygalacturonase (PG) enzyme (Schwan et al., 1997). Therefore, the isolated *Bacillus sp.* strains may contribute to better degradation of the pulp and acting in tandem with yeast as suggest by Ouattara et al. (2011). Indeed, this pectinolytic activity not only allows the emergence of useful bacterial strains by the aeration of the fermentative heaps but it also provides the necessary carbon sources for the metabolism. To this point of view, the presence of these *Bacillus* strains may allow a synergistic effect for an efficient degradation of cocoa pulp during fermentation.

Table 2. Nature and level of pectinolytic enzymes produced in both solid and liquid environments by isolated *Bacillus* strains

		P. S. I.	P. S. A. C.	P. A. S. C. D.
Total of *Bacillus* strains		432	319	163
Halo diameter(cm)	Pectinase	1 - 3.4	1 - 3.2	1 - 2.9
	PG	1 - 3.2	1 - 3	1 - 2.8
Enzymes production (µmol/min/mg of proteins)	Pectinase	0.13±0.00 - 1.26±0.01	0.13±0.00 - 1.14±0.05	0.13±0.00 - 0.94±0.00
	PG	0.09±0.00 - 0.75±0.01	0.09±0.00 - 0.67±0.04	0.09±0.00 - 0.51±0.00

Note: PG means polygalacturonase. P. S. I.: Pectinolytic Strains Isolated; P. S. A. C.: Pectinolytic Strains with Acidifying Capacity; P. S. A. C. D.: Pectinolytic Acidifying Strains with Citrate Degradation.

Additional activity of Bacillus sp. pectinolytic strains isolated: acidify capacity and citrate degradation

Many studies have clearly emphasized the ability of *Bacillus sp.* strains isolated from cocoa fermentation to express other metabolic activities such as citrate metabolism and acidifying capacity (Kouame et al., 2015a; Kouame et al., 2015b). These properties play an important role in the fermentation process. So biochemical characterization of pectinolytic *Bacillus sp.* strains previously isolated showed that among the 432 pectinolytic isolates, 51.10% display both, ability to acidify the medium and to degrade the citrate (Table 2). These isolates maintained their pectinolytic activities between 0.13±0-0.94±0.03µmol/min/mg of proteins for

pectinase and 0.09±0-0.51±0µmol/min/mg of proteins for polygalacturonase (PG) in liquid medium.

These acidifying strains may be useful for various enzymatic activities as developing. The characteristic aroma and flavours of cocoa and chocolate (Schwan and Wheals, 2004; Thompson et al., 2007), the same way as the acetic bacteria. Similarly, by their ability to degrade citrate, these *Bacillus sp.* pectinolytic strains may also contribute to modulate the pH of the medium (Schwan and Wheals, 2004), necessary for the emergence and growth of beneficial bacteria as Yeasts and Lactic Bacteria. To this regards, an absence of *Bacillus sp.* during the cocoa beans fermentation may be a limiting factor for this process.

Influence of temperature and pH on pectinolytic enzymes production by Bacillus strains

The ability of the 163 selected strains to maintain their enzymes production capacity at temperatures up to 50°C is depicted in Table 3. Only 22 of them maintain their enzymes production under 45°C of temperature unlike the 141 other strains.

Table 3. Effect of temperature on pectinolytic enzymes production from pectinolytic acidifying strains with citrate degradation

		30°C	35°C	40°C	**45°C**	50°C
Number of strains maintaining pectinolytic enzymes production		163	163	53	**22**	12
Halo diameter (cm)	Pectinase	1 - 2.9	1 - 2.9	1 - 2.9	**1 - 2.9**	2.2 - 2.7
	PG	1 - 2.8	1 - 2.8	1 - 2.8	**1 - 2.8**	2.0 - 2.5
Enzymes production (µmol/min/ mg protein)	Pectinase	0.13±0.00 - 1.26±0.01	0.13±0.00 - 1.26±0.02	0.13±0.00 - 1.26±0.02	**0.13±0.00 - 1.26±0.01**	0.15±0.00 - 1.10±0.02
	PG	0.09±0.00 - 0.75±0.01	0.09±0.00 - 0.75±0.01	0.09±0.00 - 0.75±0.01	**0.09±0.00 - 0.75±0.01**	0.10±0.01 - 0.30±0.00

Note: PG means polygalacturonase

Cocoa fermentation is generally characterized by high temperature up to 45°C occurring at 48-72 h of fermentation (Lima et al., 2011; Ho et al., 2014). As *Bacillus sp.* is known to be permanent throughout cocoa beans fermentation (Ardhana and Fleet, 2003), these 22 heat-resistant strains may therefore maintain their pectinolytic activity during these periods of high variation of temperature.

In addition, the rate of enzymes produced by these heat-resistant strains in liquid medium varied from strain to strain. These rates ranged between 0.13±0 to 1.26±0.01µmol/min/mg of proteins for pectinase and between 0.09±0 to 0.75±0.01µmol/min/mg of proteins for polygalacturonase (PG) production (Table 3). In view to these results we can assess that these heat-resistant strains were able to maintain a satisfactory level of enzymes production in these conditions of temperature fluctuation. On the other hand, the effect of pH variations on these 22 heat-resistant strains, showed that only 10 of them were able to maintain continuously their pectinolytic enzymes production for pH variations ranging from 4 to 10.

Regarding the rate of different pectinolytic enzymes produced by these 10 pectinolytic *Bacillus sp.* strains, the enzymes production is ranged between 0.22±0.04 to 0.76±0.06µmol/min/mg of proteins for pectinase production and 0.29±0.04 to 0.51±0.05µmol/min/mg of proteins for polygalacturonase (PG) (Table 4). This finding pointed out the fact that these *Bacillus sp.* strains do not have the same capacity of adaptation to temperature and pH fluctuations of the fermentative heaps. Thus, these strains were classified based on their ability to degrade pectin which is, contrary to the polygalacturonic acid, one of the major constituent of cocoa beans. At the end of this ranking, only four of them namely T_9G_4, $T_{11}I_{10}$, T_3D_{23} and T_4A_{44} were of interest for the fermentation process as potential starter.

Table 4. Ranking of pectinolytic *Bacillus* strains according to pectinase production capacity.

Strains	Pectinase (µmol/min/mg of proteins)	Polygalacturonase (µmol/min/mg of proteins)
$T_{11}I_{10}$	**0.76±0.06^{b}**	**0.51±0.05^{a}**
T_3D_{23}	**0.55±0.06^{c}**	**0.51±0.02^{a}**
T_4A_{44}	**0.45±0.04^{d}**	**0.44±0.01^{b}**
$T_9G_{4'}$	**0.44±0.05^{d}**	**0.43±0.01^{b}**
T_4D_{28}	0.38±0.02^{e}	0.29±0.04^{e}
T_1D_8	0.36±0.02^{ef}	0.34±0.0^{d}
T_5I_6	0.36±0.02^{ef}	0.20±0.02^{f}
$T_{11}I_{15}$	0.35±0.05^{f}	0.30±0.04^{e}
T_3G_3	0.25±0.05^{g}	0.40±0.05^{c}
T_3D_{14}	0.22±0.04^{g}	0.36±0.04^{d}

Note: Data are represented as means ± SEM (n=3). Mean with different letters in the same row are statistically different ($p < 0.05$) according to Duncan's test. **Legend**: In bold, four *Bacillus* sp strains with highest values of pectinase and polygalacturonase activities.

16S rRNA gene PCR and identification of isolates

Amplification of hypervariable regions (V_1, V_2 and V_6) of the 16S gene of four potential starters *Bacillus* by colony PCR yielded a DNA fragment of about 500bp (Figure 1). The sequencing analysis of amplified hypervariable regions showed that T_9G_4 and T_3D_{23} isolates had 483 bp, $T_{11}I_{10}$ and T4A44 had 493 bp and 484 bp, respectively. Analysis of these DNA fragments in the NCBI data bank has enabled us to identify these four *Bacillus* isolates as belonging to the species *thuringiensis* with 99% of similarity.

This species has also been isolated from the natural fermentation of cocoa in Ivory Coast by Ouattara et al. (2011). It is widely used in food industry for its ability to produce extracellular enzymes such as amylases, chitinases and pullunases (Thamthiankul et al., 2001) and non-food agricultural biopesticide (Sharma et al., 2002).

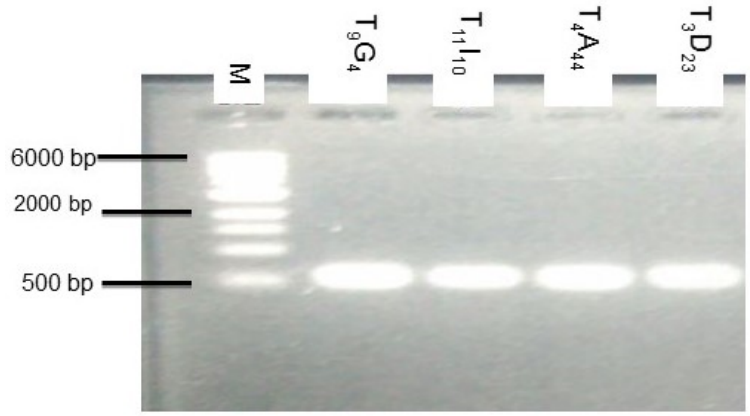

Figure 1. Electrophoretic profile of the PCR products of the hypervariable region of the 16S rRNA gene from the four potential starters *Bacillus.* **M:** molecular weight marker (ladders)

Influence of additional stress on pectinolytic enzymes production by Bacillus sp.

Temperature and pH variations are not the sole fermentative conditions that strongly influence the strains performance. Thus, the four *Bacillus sp.* strains previously selected and identified as *Bacillus thuringiensis* were tested to assess the influence of key parameters such as acids (acetic, lactic and citric) and alcohol (ethanol). The ability to maintain their pectinolytic enzymes production was also evaluated.

Acids effect on pectinolytic enzymes (pectinase and polygalacturonase) production

In acid conditions induced by lactic, acetic and citric acids, the four selected *Bacillus* strains proved to be able to maintain their potential of enzymes production

but for a low range of concentrations for lactic and acetic acids. Polygalacturonase (PG) and pectinase production occurred in medium containing lactic and acetic acid in the same range 0-0.2% for the four strains of *Bacillus thuringiensis* (Figures 2 and 3). Above these acid concentrations, *Bacillus* isolates were not able to produce these enzymes. The influence of citric acid on enzymes production by *Bacillus* was lesser than the other tested acids. Enzymes production such as polygalacturonase (PG) and pectinase was constant with increasing of citric acid concentrations in a range of 0-0.8%. The pectinolytic enzymes production decreased until 1% of citric acid (Figure 4).

The effect of acids results in a reduction of cytoplasmic pH that is likely to impair enzymes production (Cotter and Hill, 2003). This indicates that, compounds present at variable concentrations in the cocoa pulp such acids may be limiting factors for enzymes production in *Bacillus* during cocoa fermentation. However, with the ability to maintain their enzymatic performance despite these pH fluctuations throughout cocoa beans fermentation, all the four strains (T_9G_4, $T_{11}I_{10}$, T_3D_{23} and T_4A_{44}) should be particularly interesting as starters.

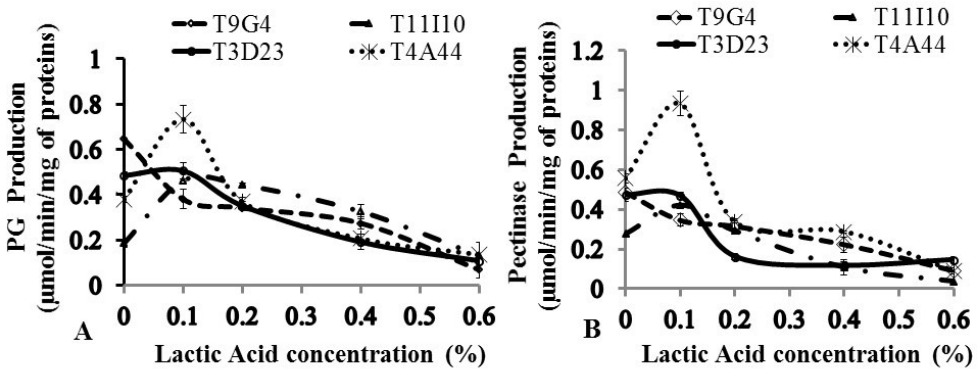

Figure 2. Effect of lactic acid on polygalacturonase (A) and pectinase (B) production by the four selected pectinolytic *Bacillus* strains

Ethanol effect on pectinolytic enzymes (pectinase and polygalacturonase) production

The influence of ethanol concentration on polyglacturonase (PG) and pectinase production is shown in Figure 5 (A and B). The results suggested that, all the four studied *Bacillus* strains maintained a good level of pectinolytic enzymes production between 0 and 8% of ethanol concentration.

This enzymes production is ranged between 27-50% for polygalacturonase (PG) after having expressed their maximum of enzymes production between 2% of ethanol for T_4A_{44} and 4% of ethanol for T_9G_4, $T_{11}I_{10}$ and T_3D_{23} (Figure 5 A). For pectinase production at the same concentrations, these strains also maintained about 21-87% of their potential production after having expressed their maximum

of enzymes production at 4% of ethanol for T_9G_4, T_4A_{44}, $T_{11}I_{10}$ and T_3D_{23} (Figure 5 B).

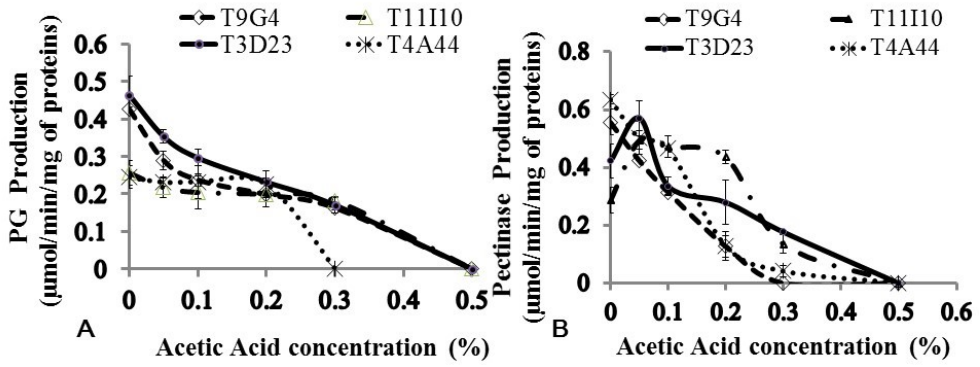

Figure 3: Effect of acetic acid on polygalacturonase (A) and pectinase (B) production by the four selected pectinolytic *Bacillus* strains

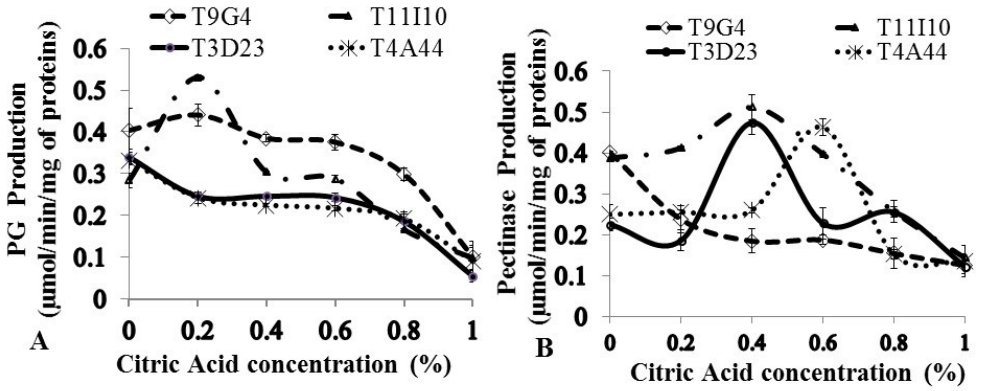

Figure 4. Effect of citric acid on polygalacturonase (A) and pectinase (B) production by the four selected pectinolytic *Bacillus* strains

These results are interesting because ethanol is a major metabolite of cocoa pulp fermentation (Roelofsen, 1958) and the maximum rate of ethanol produced by yeasts is around 8% during this process (Camu et al., 2008; Lefeber et al., 2012; Ho et al., 2014).

So, these isolates could be used in combination with high ethanol producing yeast isolates to improve the final fermenting cocoa quality.

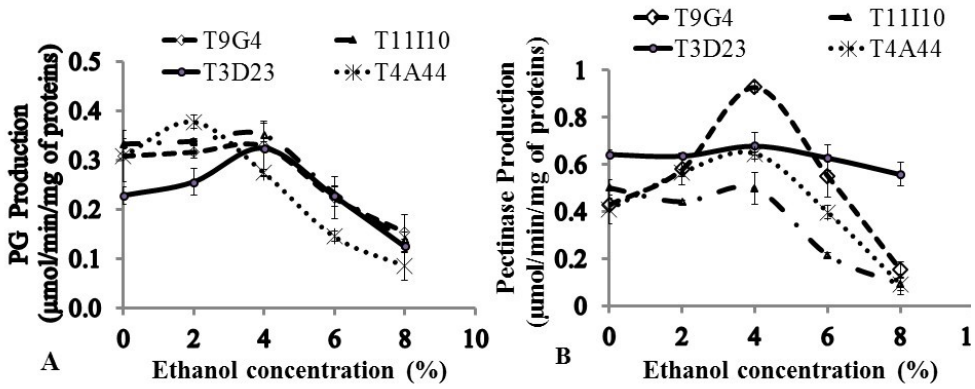

Figure 5. Effect of ethanol on polygalacturonase (A) and pectinase (B) production by the four selected pectinolytic *Bacillus* strains

Conclusions

In this study, four *Bacillus sp.* bacteria (T_9G_4, $T_{11}I_{10}$, T_3D_{23} and T_4A_{44}) involved in Ivorian cocoa fermentation were identified as *thuringiensis* species. These bacteria presented the best technological capacity: mainly pectinolytic enzymes production and in a lesser extent, acidification capacity and citrate degradation. All of them were able to maintain their enzymes production under fermentative stress conditions. That make them candidate as starter for cocoa fermentation control as these properties are very relevant and necessary for a fine and well-performed process of cocoa fermentation and production of high quality chocolate.

References

Afoakwa, O.E. Kongor, J.E. Takrama, J.F., Budu, A.S. 2013. Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. *International Food Research Journal*, **20**, 1215-1222.

Ardhana, M., Fleet, G. 2003. The microbial ecology of cocoa bean fermentations in Indonesia. *International Journal of Food and Microbiology*, **86**, 87-99.

Aydin, Y.A., Aksoy, D.N. 2009. Isolation of cellulose producing bacteria from wastes of vinegar fermentation. *Proceedings of the World Congress on Engineering and Computer Science*, **8**, 978-988.

Beishir, L. 1991. Microbiology in practice: A Self-instructional laboratory course, Fifth Edition (Harper Collins: New York), 123-345.

Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M., De Vuyst, L. 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Applied and Environmental Microbiology*, **73**, 1809-1824.

Camu, N., Gonzalez, A., De Winter, T., Van Schoor, A., De Bruyne, K., Vandamme, P., Takrama, J.S., Addo, S.K., De Vuyst, L. 2008. Influence of turning and environmental

contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. *Applied in Environment and Microbiology*, **74**, 86-98.

Cotter, D.P., Hill, C. 2003. Surviving the acid test: Responses of gram-positive bacteria to low pH. *Microbiology Molecular and Biology Review*, **67**, 429-453.

Hansen, C.E. Delolmo, M., Burri, C. 1998. "Enzyme activities in cocoa beans during fermentation". *Journal of Science and Food Agricultural*, **77**, 273-281.

Hashim, P., Selama, T.J., Muhammad, S.K.S., Ali, A. 1998. Effect of mass and turning time on free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *Journal of Science and Food Agricultural*, **78**, 543-550.

Ho, V.T.T., Zhao, J., Fleet, G. 2014. Yeasts are essential for cocoa bean fermentation. *International Journal of Food and Microbiology*, **174**, 72-87.

Kouame, L.M., Koua, G.A.Y., Adom, N.J., Goualié, B.G., Niamke, S.L. 2015a. Cocoa fermentation from Agnéby-Tiassa; Biochemical study of microflora. *American Journal of BioScience*, **3**, 203-211.

Kouame, L.M., Doue, G.G., Adom, N.J., Ouattara, H.G., Niamke, S.L. 2015b. Biochemical characterization of microbial populations involved in Loh-Djiboua cocoa's fermentation in Cote d'Ivoire. *Food and Environment Safety*, **14**, 196-205.

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., De Vuyst, L. 2012. On-farm implementation of starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolate produced thereof. *Food and Microbiology*, **30**, 379-392.

Lima, R.L.J., Almeida, M.H., Nout, M.J., Zwietering, M.H. 2011. *Theobroma cacao* L., the food of the gods: Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. *Critical Review in Food Science and Nutrition*, **51**, 731-761.

Lowry, O.H., Rosebrough, N.J., Farr, L.A., Randall, R.J. 1951. Protein measurement with Folin-phenol reagent. *Journal of Biological and Chemistry*, **193**, 265-275.

Minjares-Cassanco, A., Trejor-Aguillas, B.A., Aguilar, G. 1997. Physiological comparison between pectinase producing mutants of *Aspergillus niger* adopted either to solid state fermentation or submerged fermentation. *Enzyme Microbiology and Technology*, **21**, 26-27.

Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Anderson, T.S., Holzapfel, W.H. 2007. The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture-independent methods. *International Journal of Food Microbiology*, **114**, 168-186.

Nielsen, D.S., Hønholt, S., Tano-Debrah, K., Jespersen, L. 2005. Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). *Yeast*, **22**, 271-284.

Ouattara, H.G., Ban-Koffi, L., Karou, G.T., Sangare, A., Niamke, L.S., Diopoh, J.K. 2008. Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation. *World Journal of Microbiology and Biotechnology*, **24**, 1753-1760.

Ouattara, H.G., Reverchon, S., Niamke, S.L., Nasser, W. 2011. Molecular identification and pectate lyase production by *Bacillus* strains involved in cocoa fermentation. *Food Microbiology*. **28**, 1-8.

Passos, F.M.L., Silva, D.O., Lopez, A., Ferreira, L.F., Guimarães, W.V. 1984. Characterization and distribution of lactic acid bacteria from traditional cocoa bean fermentations in Bahia. *Journal of Food Science*, **49**, 205-208.

Pereira, G.V.M., Magalhães, K.T., Almeida, E.G., Coelho, I.S., Schwan, R.F. 2013. Spontaneous cocoa bean fermentation carried out in a novel-design stainless steel tank: Influence on the dynamics of microbial populations and physical–chemical properties. *International Journal of Food Microbiology*, **161**, 121-133.

Roelofsen, P.A. 1958. Fermentation, drying and storage of cacao beans. *Advanced in Food Research*, **8**, 225-296.

Rooijacker, J., Kamphui, H.J., Zwietering, M.H., Nout, M.J.R. 2012. Microbiota dynamics and diversity at different stages of cocoa bean industrial processing to cocoa powder. *Applied of Environmental Microbiology*, **78**, 2904-2913.

Samagaci, L., Ouattara, H.G., Goualie, B.G., Niamke, S.L. 2014. Growth capacity of yeasts potential starter strains under cocoa fermentation stress conditions in Ivory Coast. *Emirates Journal of Food Agricultural*, **26**, 861-870.

Schwan, R.F., Rose, A.H., Board, R.G. 1995. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. *Journal of Applied in Bacteriology Symposium Supplement*, **79**, 96-107.

Schwan, R.F. 1998. Cocoa fermentation conducted with a defined microbial cocktail inoculums. *Applied of Environmental Microbiology*, **64**, 1477-1483.

Schwan, R.F., Cooper, M.R., Wheals, A.E. 1997. Endopolygalacturonase secretion by *Kluyveromyces marxianus* and other cocoa pulp-degrading yeasts. *Enzyme Microbiology Technology*, **21**, 234-244.

Schwan, R.F., Wheals, A.E. 2004. The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Review of Food Science and Nutrition*, **44**, 205-221.

Sharma, H.C., Ortiz, R. 2002. Host plant resistance to insects: an eco-friendly approach for pest management and environment conservation. *Journal of Environment Biology* **23**, 111-135.

Soares, M.M.C.N., Da Silva, R., Gomes, E. 1999. Screening of bacterial strains for pectinolytic activity: characterization of the polygalacturonase produced by *Bacillus* sp, *Revista de Microbiologia*, **30**, 299-303.

Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S., Panbangred, W. 2001. Chitinase from *Bacillus thuringiensis* subsp. Pakistanian. *Applied in Microbiology and Biotechnology*, **56**, 395-401.

Thompson, S.S., Miller, K.B., Lopez, A.S. 2001. Cocoa and coffee, *Microbiology and Food Fundamentals Frontiers*, **35**, 721-733.