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ORIGINAL RESEARCH PAPER

GROWTH CAPACITY OF *BACILLUS* POTENTIAL STARTER STRAINS ISOLATED FROM COCOA BEANS FERMENTATION UNDER CULTURE STRESS CONDITIONS

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Abstract: The study investigates the growth capacity of *Bacillus* with pectinolytic activity, acidifying and citrate metabolism capacities under culture stress conditions. Spontaneous heaps fermentation was conducted from cocoa of six producing regions of Côte d'Ivoire. *Bacillus* isolated using plate agar on nutrient medium were analyzed for pectinolytic enzymes production, citric acid breakdown, acidification and growth capacity under different stress conditions. A total of 970 *Bacillus* strains were isolated and 44.53 % of them produced pectinolytic activity. Among pectinolytic strains, 163 (37.73 %) exhibited acidifying and citrate metabolism capacity. Five (5) of these strains exhibited a strong thermotolerance at 50 ºC with the optimal growth at 35 ºC and a good capacity to grow at *p*H 4 to *p*H 8. Moreover, sugar concentrations ranged 5 to 25 % showed low effect on all tested strains growth with a maximum growth at 5 % fructose and sucrose concentration and at 15% glucose concentration. However, ethanol stress conditions (up to 8 %) repress strongly growth capacity of the strains analyzed. This study indicates that *Bacillus* strains involved in Ivorian cocoa fermentation possess some properties essential for a well-fermented cocoa. Therefore, these results show that *Bacillus* studied should be potential candidate as starter for cocoa beans fermentation control.

Keywords: *Bacillus, cocoa beans fermentation, stress conditions, techno functional properties*

INTRODUCTION

Cocoa beans are the principal raw material for chocolate production. However, their quality mainly depends of postharvest processing such as fermentation and drying [1, 2]. Cocoa fermentation is crucial for the development of quality beans and chocolate thereof. The fermentation of cocoa is a spontaneous process performed by a whole microorganism, including essentially fungal organisms (yeasts) and acetic acid bacteria and lactic acid bacteria [3]. During fermentation of the pulp that surrounds the cocoa beans, yeasts produced pectinolytic enzymes and ethanol. As concerning acetic acid and lactic acid bacteria, they produced acetate and lactic acid. These acids and a part of ethanol then diffuses into the beans [4, 5], where it initiates a cascade of chemical and biochemical reactions leading to precursor molecules for cocoa flavor [2 – 4]. At days, the role of *Bacillus* in this process remains unclear. However, these strains were reported to be implicated in the production of tetramethylpyrazine [6], now known as the major component of chocolate aroma [7]. Moreover, many studies have shown the ability of *Bacillus* to produce pectinolytic enzymes [8, 9].

On the other hand, cocoa fermentation remains difficult to control leading to inconsistent production of cocoa quality. In this context, many studies suggest that using of microbial starter culture could improve fermentation process [10 – 14]. Thus, some authors had used microbial cocktail consisting only of yeasts, lactic and acetic acid bacteria [15 – 17].

Absence of these bacteria in these cocktails could due to the fact that *Bacillus* are thought to be responsible for off flavor of fermented cocoa [3]. However, *Bacillus* species are part of the major natural flora of cocoa fermentation and have many technological properties essential to obtaining quality of cocoa [18]. Therefore, fermentation essays should assess the impact of these bacteria on the quality of cocoa beans. But, *Bacillus* technological properties must be studied for use as a potential starter. Previous studies were investigated influence of fermentation parameters on the production capacity of pectinolytic enzymes. Here, we analyze the ability of these strains with various technological properties to grow under different fermentative stress.

MATERIALS AND METHODS

All reagents used in this study were of analytical grade and were provided by Merck (Germany) and Sigma Aldrich (Germany).

Cocoa beans fermentation conditions and *Bacillus* strains isolation

The cocoa pods were harvested at farms from 6 production areas of Côte d'Ivoire including Agneby-Tiassa (6°00' North 4°00' West); Sud-Comoé (5°30′ 0 North 3°15′ West), Loh-Djboua (5° 40′ North 5° 30′West), Guemon (6° 44′ 00″ North 7° 21′ 00″ West), Nawa (5° 47′ 00 North 6° 36′ 00"West) and Indénié-Djuablin (6° 43′ 47″North 3° 29′47″ West). The spontaneous cocoa bean fermentation was performed in National Flowers Center of the university Félix Houphouët-Boigny in traditionally conditions by heap fermentation during 6 days.

At the start of the fermentation (0 h) and after each 12 hours of fermentation, the fermenting heap was turning and 200 g of beans were collected in Stomacher bag for microbial analyzed.
Isolation and numeration of *Bacillus* sp were carried out in nutrient agar supplemented with 50 pg·mL^{-1} of nystatin (Bristol-Myers Squibb, France) as previously described [18]. Plates were incubated at 30 °C for 2 days and after incubation period, the colonies were counted (expressed as CFU per gram cocoa pulp-bean mass) [16]. The presumptive colonies on each agar plate were subcultured and identified by Gram staining reaction, and biochemical pattern [9]. All isolates were stored in nutrient medium supplemented with glycerol (Cooper, France) 20 % at −80 °C.

Screening of *Bacillus* pectinolytic activity strains

The screening of pectinolytic *Bacillus* strains was performed in solid medium containing 0.28 % NH_4SO_4, 0.6 % K_2HPO_4, 0.01 % $MgSO_4$, 0.2 % KH_2PO_4, 0.02 % yeast extract and 1.7 % agar at *p*H adjusted to 6. The medium was supplemented with 1 % pectin as sole carbon source [8]. Inoculation of the isolates was carried out as previously described by Yao *et al.* [9] and the plates were incubated at 30 °C during 24 to 48 h. After incubation, the clear zones around the wells, indicating pectinolytic activity were revealed with a solution of iodine and potassium iodide (5 g potassium iodide + 1 g iodine + 330 mL distilled water) as described by Soares *et al*. [19].

Acidification capacity of *Bacillus* strains

Evaluation of acidification capacity of *Bacillus* strains was performed in HS medium (2 % glucose, 0.3 % peptone, 0.5 % yeast extract, 1.5 % $CaCO_3$, 1.2 % agar) supplemented with 0.0016 % bromocresol green as color indicator [20]. Thereby, each isolate was cultured in 4 mL of the medium contained in 10 mL tubes and incubated at 30 °C during 48 hours. Acid production was monitored by formation of yellow area in the tube with or no gas production [9].

Citrate metabolism capacity of *Bacillus*

The citrate metabolism capacity was evaluated on citrate of Simmons solid medium containing sodium citrate (0.1%) as sole carbon source. After sterilization of the medium, bacterial strains were cultured on sloping citrate agar and incubated at 30 °C for 24 hours. The citrate metabolism was monitored by the presence of colonies on the plate with or no agar turn from green to blue after incubation [21].

Effects of temperature and *p*H on *Bacillus* growth

Effects of temperature and *p*H on the growth of *Bacillus* strains were analyzed in liquid medium as described by Sow *et al.* [22]. Therefore, pure culture of *Bacillus* was suspended in a sterile saline solution to give an optical density of 1 at 600 nm and then 200 µL of this suspension were added in 5 mL of nutrient broth. The cultures were incubated at different temperatures from 30 to 50 °C during 48 hours [9]. Concerning the influence of *p*H, nutrient broth was prepared at various *p*H values (3 to 10) and

200 µL of bacterial suspension (optical density of 1 at 600 nm) were used to inoculate 5 mL of broth at different *p*H and incubated at 30 °C. After incubation, the absorbance was measured at 600 nm against the sterile nutrient broth by using of spectrophotometer (Pioway Medical Lab-UV752, Singapore).

Resistance to alcoholic and sugar stress

Evaluation of resistance to ethanol and sugar stress was carried out on YEP liquid medium as described by Samagaci *et al*. [23]. To prepare the stress medium, the YEP medium (0.1 % yeast extract and 0.3% casein peptone) were cooled after autoclaving and maintained in liquid state at 45 °C in water bath (Julabo TWB12, Germany) and then appropriate quantity of alcohol was aseptically added to the medium to obtain the fixed concentration. Ethanol concentrations were added with different final concentrations (v/v) ranging from 1 to 12 %. Concerning the effect of sugar on *Bacillus* growth, the medium was supplemented with tested sugar (glucose, fructose or sucrose) at different concentrations (5 % to 25 %) before sterilization. For the both stress factors, 5 mL of the medium is inoculated with 200 µL of *Bacillus* cells pre-culture (OD600 = 1) and incubated at 30 °C for 48 hours. The negative control was prepared in the same conditions except that it did not contain the corresponding compound studied. The absorbance was measured at 600 nm against the sterile YEP medium.

RESULTS AND DISCUSSION

Technological properties of *Bacillus* strains

A total of 970 *Bacillus* strains were isolated from fermenting cocoa beans of the six regions and analyzed for pectinolytic activity and both acidifying and citrate metabolism capacities. The Table 1 shows proportion of *Bacillus* isolates exhibit pectinolytic activity, acidifying and citrate metabolism capacities depending on the region. Among these isolates, 432 (44.53 %) were able to produce pectinolytic enzymes. The regions of Sud-Comoé and Indénié-Djuablin recorded the most important rate of pectinolytic isolates with 64.60 % and 63.78 %, respectively. Pectinolytic activity is essential for the obtention of well fermenting cocoa. Indeed, pectinolytic enzymes breakdown of the cocoa pulp that allow the aeration of cocoa fermenting mass for the growth of acetic acid bacteria that are the main actors responsible for the raise of temperature and decrease of inner *p*H of cocoa beans. These biochemical changes promote generation of molecule precursors of cocoa aroma. In addition, pectinolytic activity provide carbon source from pectin for the growth of the microflora. Therefore, *Bacillus* plays a crucial role in cocoa fermentation by production of pectinolytic enzymes and may be complementary with yeasts for an efficient degradation of cocoa pulp during fermentation [8, 9].

Among the pectinolytic strains, a large proportion of *Bacillus* isolates up to 73.84 % possesses acidifying property (Table 1). In addition, 163 (37.73 %) strains of these microbial populations have citrate metabolism capacities (Table 1). It was also observed that the distribution of these strains is not uniform in the six regions that indicating the local geographic area could influence the composition of the microflora involved in cocoa fermentation [3]. Acidification is one of the most important properties desired in

cocoa fermentation. In fact, acids produced by microbial metabolism diffuse into beans and contribute to activate several enzymatic activities which lead to the formation of characteristic aroma and flavor of cocoa and chocolate [11, 24]. Although our strains acidification capacity seemed to be an interesting technological property, they exhibit a very weak acidifying activity in comparison with that of acetic and lactic acid bacteria. Therefore, *Bacillus* involved in cocoa fermentation may not play a significant role in the acidification process of fermenting beans comparatively to acidifying strains in cocoa fermentation such as acetic acid bacteria [9].

However, it was observed that these *Bacillus* acidifying isolates were able to metabolize citrate. Yao et al. [9] also reported ability of *Bacillus* strains to metabolize citrate. The citrate metabolism is known to have a benefic effect on fermentation process. Indeed, citric acid is the compound responsible for the initial *p*H of cocoa pulp before fermentation process [25, 26]. The degradation of this acid in the first stage of fermentation allows the increase of the *p*H favorable for the growth of many microorganisms groups [3]. In addition, citric acid metabolism is also lead to produce certain molecules aroma such as acetoin which are desirable flavor in fermentation process [27]. It is well known that yeasts and lactic acid bacteria assumed the breakdown of citric acid during cocoa fermentation process. These results indicate that *Bacillus* strains capable to breakdown citrate could also involved in cocoa aroma production. Finally, the numbers 163 *Bacillus* strains with together pectinolytic activity, acidification and citrate metabolism capacities may strongly contribute to the production of well-fermented cocoa especially in fermentation assay process. However, these strains could be able to grow under certain fermentation stress conditions.

Table 1. *Distribution of isolates strains*

Regions	**Numbers of tested strains**	**Numbers of pectinolytics strains and percentage (%)**	**Numbers of pectinolytic strains with acidification and percentage (%)**	**Numbers of pectinolytic strains with acidification and citrate metabolism capacities and percentage (%)**
Lôh-Djboua	173	78 (45%)	57 (73.07 %)	40 (70 %)
Sud-Comoé	113	73 (64.6 %)	66 (90.41 %)	39 (53.42 %)
Agnéby-Tiassa	314	104 (33 %)	67 (64.42 %)	55 (52.88 %)
Guemon	122	41 (33.6 %)	32 (78.04 %)	11 (26.82 %)
Nawa	121	55 (45.45 %)	27 (49.09 %)	07 (12.72 %)
Indénié-Djuablin	127	81 (63.78 %)	70 (86.42 %)	11 (13.58 %)
Total	**970**	**432 (44.53 %)**	**319 (73.84 %)**	**163 (37.73 %)**

Influence of fermentation conditions on *Bacillus* growth

Influence of temperature

The results show that maximum growth is observed between 30 °C and 40 °C before decline for temperature up to 45 °C (Table 2). These results confirm those of Ehon *et al.* [28] which demonstrated that the *Bacillus* reach their maximum growth between 30 and

45 °C. In addition, 5 isolates showed good ability to grow at 50 °C with relative growth rates ranging between 12.98 and 64.37% (Table 2).

The increase of temperature during fermentation process is essential for obtaining quality cocoa beans. In fact, heat produced by bioconversion of ethanol in acetic acid by acetic acid bacteria [29] in combination with acidity of beans causes the death of the seed embryo as well as the end of fermentation. These biochemical changes are leading to the formation of precursor molecules that are necessary for the development of a characteristic aroma, flavor and color of beans [11, 24]. Generally, the temperature of the fermentation heaps can reach to 50 °C at 48 – 72 hours of fermentation [30, 31]. Therefore, this high temperature could be responsible for the decline of several groups of microorganisms such as yeasts, acetic acid bacteria and lactic acid bacteria [25]. Since the thermotolerance constitutes an important property during fermentation process, *Bacillus* strains with growth capacity at 50 °C were selected for further study.

Table 2. *Growth capacity of Bacillus at high temperature*

Tested strains	Absorbance (OD 600) at 30 °C	Absorbance (OD 600) at 40 °C	Absorbance (OD 600) at 45 °C	Absorbance (OD 600) at 50 °C	Relative growth at 50° C
T3G3	0.7235 ± 0.04	1.0852 ± 0.01	0.603 ± 0.0	0.144 ± 0	19 %
T2G4	0.793 ± 0.01	0.935 ± 0.01	0.303 ± 0.01	0.103 ± 0	12.98 %
T9D167	0.727 ± 0.01	1.054 ± 0.01	0.363 ± 0.01	0.175 ± 0.02	24 %
T4A44	0.567 ± 0.01	0.901 ± 0.01	0.741 ± 0.01	0.365 ± 0.02	64.37 %
T12I9	0.673 ± 0.03	0.576 ± 0.02	0.293 ± 0.0	0.125 ± 0.01	18.57 %

Abbreviations: **T**, time ; **G**, Guémon ; **D**, Loh-Djboua **; A**, Agneby-Tiassa **; I,** Indénié-Djuablin.
T3G3 for example corresponding to strain No. 3 isolated at the 3rd sampling (T = 24 hours) in the Guémon area

Influence of pH on Bacillus growth capacity

Figure 1 shows that *Bacillus* tested strains present a good capacity to grow at *p*H 4 to *p*H 8. However, no growth was observed when isolates were cultured in acid (*p*H 3) medium. In addition to *p*H values ranged to 9 – 10, the growth capacity decreases highly (Figure 1). For strains T3G3, T9D167, T4A44 and T12I9, maximum growth occurs at *p*H 8 and at *p*H 7 for the strain T2G4. The ability of these strains to growth at *p*H values ranged to 4 and 10 could explain presence of the *Bacillus* during the all step of cocoa fermentation process with *p*H values generally ranged from 3.9 to 7.9. Indeed, cocoa fermentation is assumed to be generally characterized by variation of *p*H [3]. At the beginning, the *p*H of the fermentation heap is highly acid due to the citric acid contained in the pulp. After 48 hours, the *p*H of the fermentation heap increases due to diffusion in the cotyledons of acids produced mainly by lactic and acetic acid bacteria. This increase in the *p*H of the pulp contributed to low cotyledons *p*H for activation of endogenous enzymes responsible for the synthesis of aroma precursors cocoa [3, 25]. As this variation being essential for obtaining a quality cocoa, pectinolytic *Bacillus* strains with acidification and citrate metabolism abilities and capable of withstanding this different *p*H could be interesting in a fermentation test.

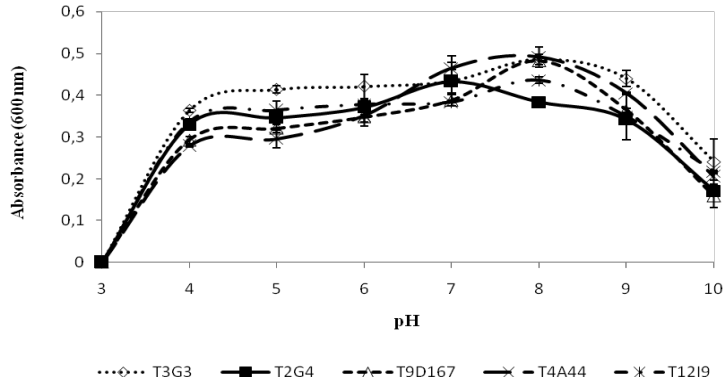

Figure 1. *Bacillus growth at different pH*

Influence of alcohol on Bacillus growth

All the five pectinolytic strains with acidification and citric metabolism capacities were analyzed for alcohol and osmotic tolerances (Figure 2). The results show that all of the tested strains supported 7 % ethanol initial concentration. However, maximum grow was observed at 4 % ethanol initial concentration for strains T3G3, T2G4 and T2I9, at 5 % for strain T4A44. It also observed a lower growth of strain T9D167 on medium containing ethanol (Figure 2). In addition, the drastic death of *Bacillus* population beyond 7 - 8 % alcohol (Figure 2), gives an insight into the pressure exerted by alcoholic stress on these strains.

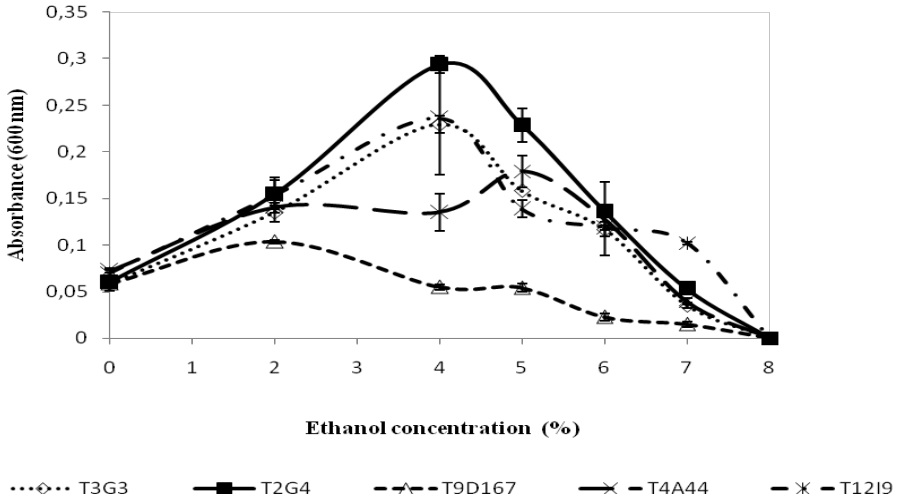

Figure 2. *Bacillus growth under alcohol stress*

In fact, ethanol is an inhibitor of growth of many microorganisms such as yeasts and lactic acid bacteria, even at relatively low concentrations, it inhibits cell division, decreases cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death [9, 32]. The maximum amount of ethanol in cocoa mass was found to be round 0.8 % (w/v) [15] and 8 % (w/w) [33]. Therefore, the growth inhibition up to 7 % ethanol presented by all the tested pectinolytic strains isolated in this work indicates that these strains are not able to survive under alcoholic

stress conditions during cocoa fermentation. These results could explain the low growth of *Bacillus* strains in the first 48 hours of spontaneous fermentation corresponding to yeasts ethanol production time [18].

Influence of sugar on *Bacillus* growth

In general, the sugar content (5 to 25 %) revealed to have a low influence on pectinolytic strains (Figure 3).

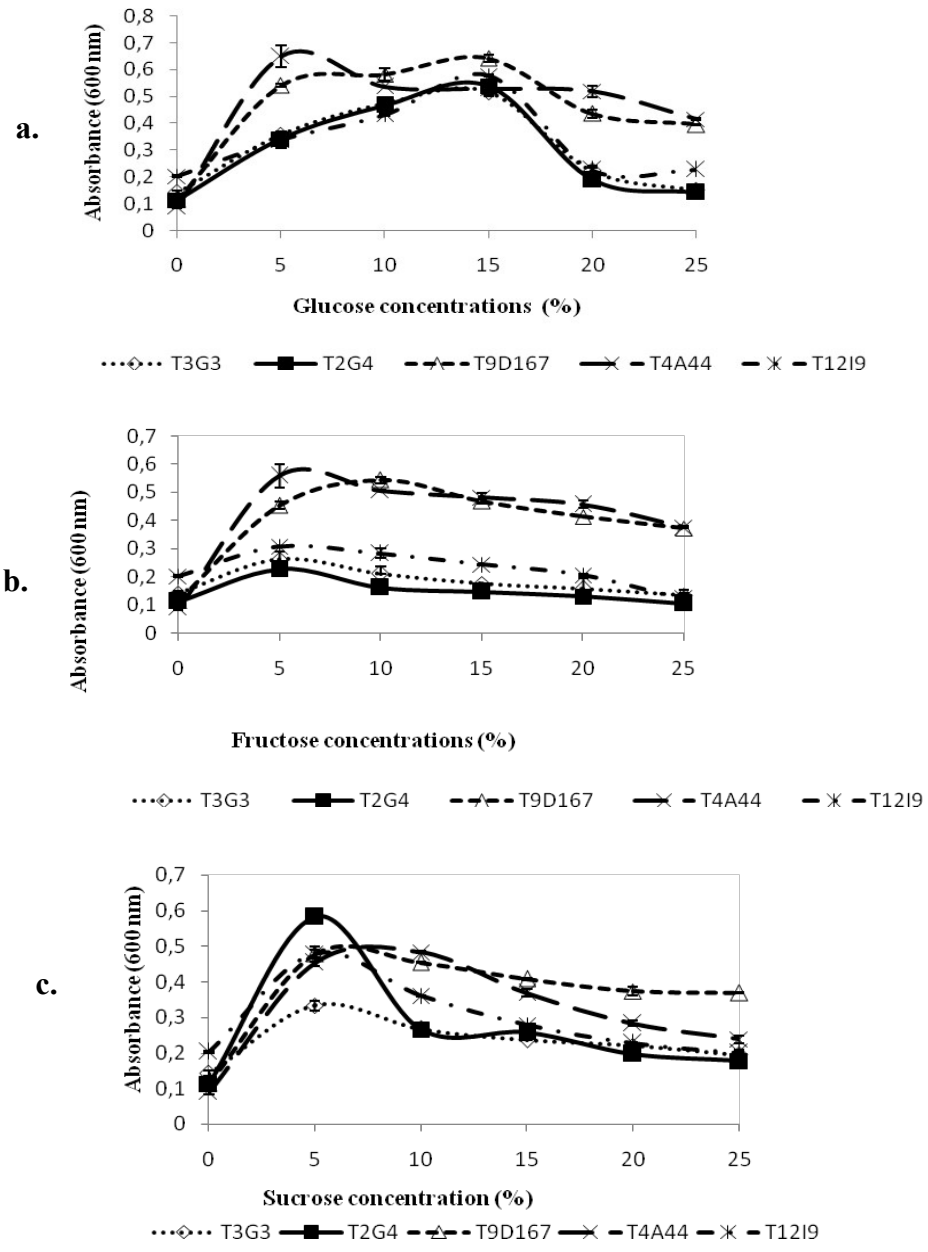

Figure 3. *Bacillus growth at different sugar concentration*

Indeed, the maximum growth was observed at 5 % sucrose and fructose concentrations, and at 15 % glucose concentration. However, up these concentrations, the sugars have low effect on tested strains (Figure 3).
Among the sugars tested, glucose seems to have the most hindering effect on growth capacity of *Bacillus* at concentration up 20 % (Figure 3a). It also observed that strains T9D167 and T4A44 present more resistance capacity to the tested sugars than T3G3, T2G4, and T12I9. The sugars influence on growth capacity may be due to osmotic pressure and could affect their general metabolism involving enzymes production, acid production and citrate metabolism [34]. However, the total concentration of sugar present in the cocoa pulp round 10-15 % [3, 35]. This indicates that, sugar present in the cocoa pulp could not limit bacteria growth capacity and their activities during cocoa fermentation. However, previous studies showed that high sugars (up 10 %) content from the pulp at the beginning of fermentation [14] affect pectinolitic enzymes production capacity [9]. In this context, pectinolytic enzymes, acids and citrate lyase production in tested strains may be more favorable in the advanced stage of cocoa fermentation when sugars content is low. In all case, strains T9D167 and T4A44 with sugar higher resistance capacity could be particularly interesting as starter to improve fermented cocoa quality.

CONCLUSIONS

In conclusion, this study showed that *Bacillus* involved in cocoa fermentation possess ability to produce pectinolytic enzymes, capacity to breakdown citric acid and to produce acid. Moreover, five of isolates were able to growth under fermentation stress conditions such as high temperature, *p*H, alcohol and sugar stress. Due these properties are very relevant and necessary for production of high quality of chocolate, this study confirms that *Bacillus* strains could play a more important role in cocoa fermentation than it is believed at date.

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