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ORIGINAL PAPER

Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation

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Abstract The role of bacilli in cocoa fermentation is not well known. Their potential of production of pectinolytic enzymes during this process was evaluated. *Bacillus* growth was monitored and pectinolytic strains were screened for their use of pectin as sole carbon source. Effects of cocoa fermentation parameters susceptible to influence on enzyme production were analysed. Among 98 strains isolated, 90 were positive for pectin degradation and 80% of them presented detectable pectinolytic activities in submerged fermentation. Forty-eight strains produced polygalacturonase (PG), 47 yielded pectin lyase (PL) and 23 strains produced both enzymes. Bacilli growth was not significantly affected during fermentation. PL production was favoured by galactose, lactose, glucose as sugars, and arginine, glutamine, cysteine and ammonium sulphate as nitrogen compounds. Pectin at low concentration (0.05%) and iron stimulated PL production. It was strongly repressed by galacturonic acid (1%), and negatively affected by nitrogen starvation, zinc and temperatures above 45°C. PL yield was very weak below pH 4.0 and in anaerobic conditions. PG production was weakened by sucrose and cation depletion. It was increased slightly by cysteine, ammonium nitrate and nitrogen starvation and significantly above 40°C. PG synthesis was not affected by acidic pH (3.0–6.0) or oxygen availability. As fermentation products, lactate and acetate lowered the production of both enzymes while ethanol had no effect. The high proportion of pectinolytic producers among the strains studied and analysis of factors influencing pectinolytic enzymes production, suggest that *Bacillus* sp. is liable to produce at least one enzyme during cocoa fermentation.

Keywords *Bacillus* · Cocoa fermentation · Pectinolytic enzymes · Potential production · screening

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Introduction

Cocoa bean and chocolate quality depend strongly on the cocoa fermentation process (Timbie et al. 1978; Gill et al. 1985; Villeneuve et al. 1989; Schwan 1998; Schwan and Alan 2004). During this process, microbial fermentation induces numerous reactions leading to a deep modification of the biochemical characteristics of beans. These complex biochemical reactions triggered mainly by yeast, moulds, *Bacillus*, acetic and lactic acid bacteria (Rombouts 1952; Ostovar and Keeney 1973; Schwan 1998) cause the f ormation of organoleptic substances (Timbie et al. 1978; Biehl et al. 1993). Among the crucial microflora, acetic acid bacteria are key microorganisms in the formation of specific precursors of cocoa aroma (Forsyth and Quesnel 1963; Jinap 1994). These bacteria are known to possess an aerobic metabolism (Yamada et al. 1999).

The aeration conditions necessary for growth of acetic acid bacteria are in part created by pectinolytic enzymes which break down the pectin responsible for the viscosity and stickiness of cocoa pulp (Schwan 2004). These enzymes allow the collapse of the pulp and the formation of void space between the beans, into which air percolates.

Pectinolytic enzymes are classified in two main groups according to their mode of attack on the pectin molecule: de-esterifying enzymes (pectin methylesterase EC 3.1.1.11) which removes the methoxy group from pectin, and depolymerases that cleave the β-(1, 4) glycosidic bonds between galacturonate units either by hydrolysis (polygalacturonase EC 3.2.1.15) or by trans-elimination (pectin lyase EC 4.2.2.10 and pectate lyase EC 4.2.2.2).

Higher pectinolytic activity during cocoa fermentation is liable to increase the fermentation speed and improve cocoa products (Freire et al. 1990). To date, attention has been focused only on yeasts (Sanchez et al. 1985; Schwan and Rose 1994; Schwan et al. 1996), which are considered to be the sole source of pectinolytic enzymes during cocoa fermentation. However, pectinolytic enzyme production by some *Bacillus* strains from soil (Soares et al. 1999; Soriano et al. 2005), water retting process (Tamburini et al. 2003) and fermented food (Oguntoyinbo et al. 2003) have been reported. This suggests that *Bacillus* present in the fermenting cocoa mass might contribute to the yield of pectinolytic enzymes, but this has not yet been reported.

The role of *Bacillus* in cocoa fermentation is not well known, so this bacterium has never been involved as starter in attempts to control the fermentation process. Such studies are judicious since they could allow the selection of additional strains for desired enhanced pectinolytic activity.

This paper evaluates the potential of production of pectinolytic enzymes from *Bacillus* sp. in the cocoa fermentation process.

Materials and methods

Cocoa pods were harvested from three cultivars (Forastero, Criollo, and Trinitario) from the CNRA experimental plot in Bingerville (Abidjan).

Fermentation conditions and *Bacillus* isolation

Bacilli were collected from three harvest seasons of cocoa and from different fermentation methods (tarpaulin, wooden box and banana leaves). The fermenting mass was about 500 kg and was constituted of mixed genotypes. The fermentations lasted six days. Strains were isolated on nutrient agar containing cycloheximide 100 mg/l to inhibit fungal growth. *Bacillus* strains were characterized as Gram- and catalase-positive rods, spore-forming and able to grow aerobically.

In the last season a monitoring of different fermentations was carried out. The size of the *Bacillus* population was regularly followed during the fermentation by performing an enumeration using the decimal dilution method. The pH and temperature were also recorded directly at 15 cm depth on the fermenting heap, with portable pH-meter and thermometer.

Screening of *Bacillus* strains for pectinolytic activity

Pectinolytic strains were screened using an adapted method of Soares et al. (1999). Screening was performed on solid medium at pH 6.0 containing 1% citrus pectin with 68% methoxylation (Sigma), 0.14% $(NH_4)_2SO_4$, 0.20% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$ and 0.10% nutrient solution (containing per l) 5 mg $FeSO_4{\cdot}7H_2O$; 1.6 mg $MnSO_4{\cdot}H_2O$; 1.4 mg $ZnSO_4{\cdot}7H_2O$; 2.0 mg $CoCl_2$). Five wells (0.3 cm diameter 2 mm deep) were made aseptically in the solid medium. Then, pure culture was suspended in a salt solution 0.6% (w/v) to give an optical density of 1 at 421 nm. The wells were loaded with 7 µl of the suspension. All the wells of a given medium were seeded with a single bacterial strain. After 48 h incubation at 30°C, the solid medium was flooded with iodine–potassium iodide solution to detect clearance zones corresponding to enzymatic activity.

Culture condition and enzyme production

Bacillus strains presenting clearances zones round the wells, were used for enzyme production assays. Liquid medium containing 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4{\cdot}7H_2O$, pH 6.0, was autoclaved for 15 min at 121°C. After cooling at room temperature, the medium was inoculated with 1.0 ml of bacterial suspension prepared as described above. In aerobic conditions, cultures were grown in 125-ml Erlenmeyer flasks with 25 ml of medium in a rotary shaker (150 rev min^{-1}) at 30°C for 48 h. Anaerobic conditions were produced by layering the medium with paraffin oil, without shaking. Fumarate (0.25%) was added to the medium as an electron acceptor. Biomass was separated by centrifugation at 1000 *g* for 20 min at 4°C. Enzyme activities were measured in the cell-free supernatant.

Protein and enzyme assays

Polygalacturonase (PG) activity was determined spectrophotometrically (spectronic Genesys) by measuring the release of reducing groups from apple pectin using the 3,5-dinitrosalicylic acid (DNS) reagent assay (Miller 1959). Galacturonic acid monohydrate was used as standard. The reaction mixture containing 1 ml 1% apple pectin in 0.1 M acetate buffer, pH 6.0 and 0.5 ml of crude enzyme solution,

was incubated at 40°C for 20 min. The blank was prepared in the same way except that the crude enzyme was heated at 100°C for 20 min. One unit of enzymatic activity (U) was defined as the amount of enzyme which releases one μmol of galacturonic acid per minute under assay conditions.

Pectin lyase (PL) activity was determined by measuring the increase in absorbance at 235 nm of substrate solution (1 ml 1% apple pectin in 0.1 M tris–HCl buffer 1 mM $CaCl_2$ $2H_2O$, pH 8.5) mixed with 0.25 ml enzyme solution, at 40°C for 20 min. One unit of enzymatic activity (U) was defined as the amount of enzyme which released 1 μmol of unsaturated uronide per minute, based on the molar extinction coefficient ($\varepsilon = 5500\ l\ mol^{-1}\ cm^{-1}$) of the unsaturated products (Albersheim 1966).

Pectin methylesterase activity was evaluated by recording the pH decrease of the medium with a microprocessor pH meter (Hanna instruments, model pH 213), and by the titration of carboxylic groups released with 1 mM NaOH. The medium consisted of 3 ml of enzyme preparation added to 3 ml of 1% apple pectin, pH was adjusted to 7.2. The reaction mixture was incubated at 30°C; the decrease of pH against the blank was recorded regularly at 20 min intervals.

Protein content was determined by the Bradford method using bovine serum albumin as standard.

All the experiments were realized in triplicate.

Choice of parameters studied

Temperature, pH, sugar and pectin content, nitrogen compounds, oxygen availability, metal ion, and microbial metabolism products such as lactate, acetate and ethanol were the relevant parameters chosen to study the ability of the strain to produce enzymes based on the characteristics of cocoa pulp and fermentation conditions.

Results

Cocoa fermentation and *Bacillus* growth

At the beginning of the fermentation, in a period stretching from 0 to 36 h there was a regular increase of cocoa mass temperature which did not exceed 36°C in all fermenters. Beyond 36 h a thermal fluctuation occurred during the remaining time of fermentation. Globally, the temperature of fermenting mass ranged from 27 to 50°C (Fig. 1). Cocoa mass was also subjected to pH fluctuation between 3.5 and 5.5 (Fig. 2). *Bacillus* population was present at the first stage of fermentation with about 10^5 c.f.u.g^{-1} and remained nearly static during fermentation. However bacilli growth rate tends to increase in the latter stage of fermentation (Fig. 3).

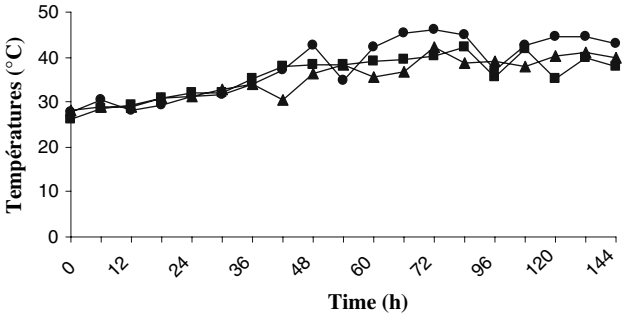

Fig. 1 Temperature variations of cocoa mass during fermentation. (-▲-) Tarpaulin; (-•-) wooden box; (-■-) banana leaves

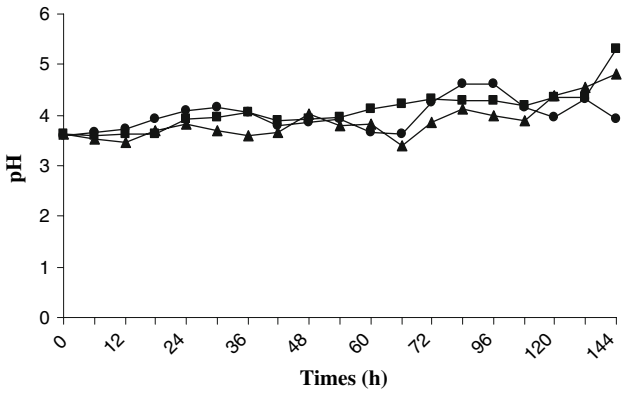

Fig. 2 pH variation of cocoa mass during fermentation (-▲-) Tarpaulin; (-•-) wooden box; (-■-) banana leaves

Screening of pectinolytic strains

Ninety-eight *Bacillus* strains were isolated and assayed for pectinolytic activity and 90 were positive for pectin degradation in plate assay as evidenced by the clear halo (data not shown). Among them, 72 were able to produce

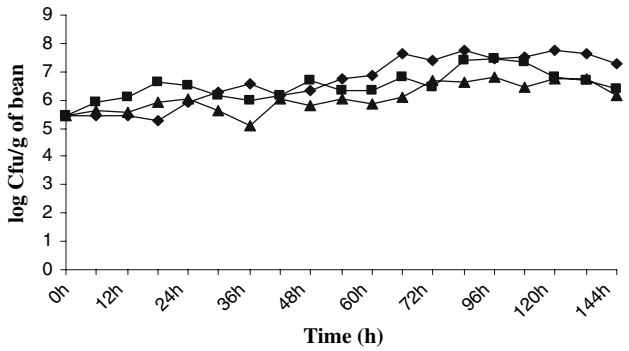

Fig. 3 Evolution of *Bacillus* population during cocoa fermentation. (-▲-) wooden box; (-•-) Tarpaulin; (-■-) banana leaves

detectable enzyme activity when cultivated in liquid medium (submerged fermentation). Polygalacturonase was produced by 48 strains and 47 were able to produce pectin lyase while 23 strains produced both types of enzyme. A very weak pectin methylesterase activity was detected with three strains. Thus only polygalacturonase and pectin lyase were further studied.

PL production

To analyse the conditions of enzyme production, *Bacillus* sp. BS-66 among PL producers was randomly chosen as model. Among the carbon sources studied, lactose, galactose and to a less extent glucose, promoted PL production. The yield reached was double with lactose and galactose. Galacturonic acid strongly decreased the production of PL, 4-fold lower than the control (Table 1). The same effect was observed with pectin (Fig. 4). The others carbon sources did not affect significantly PL synthesis.

In nitrogen starvation conditions, a strong decrease of PL production was also observed (Table 2), so all the nitrogenous compounds studied increased PL yield. The highest production was reached with arginine, cysteine and glutamine, increasing the amount of PL about 18-fold while tryptophan and yeast extract proved to be less effective.

The presence of metal ions, such as magnesium, zinc and manganese, at 0.01% concentration in the culture medium, strongly reduced PL production, the effect being more pronounced with zinc (Table 3). In contrast, iron stimulated the production of PL.

Table 1 Effect of various sugars on the yield of pectinolytic enzymes

	Enzyme production (U/mg of protein/ml of supernatant)	
Sugars	PL (*Bacillus* sp. BS-66)	PG (*Bacillus* sp. BS-6)
Negative control	3.52 (±0.38)	1.03 (±0.13)
Fructose	3.74 (±0.07)	1.11 (±0.24)
Sucrose	2.76 (±0.10)	0.01 (±0.00)
Glycérol	2.23 (±0.16)	–
Mannose	4.68 (±0.73)	0.89 (±0.01)
Galactose	7.54 (±0.69)	1.01 (±0.23)
Lactose	7.12 (±1.05)	0.81 (±0.21)
Xylose	4.24 (±0.42)	0.96 (±0.07)
Ribose	3.30 (±0.50)	1.35 (±0.26)
Cellobiose	2.81 (±0.24)	1.70 (±0.50)
Glucose	5.71 (±0.58)	1.22 (±0.28)
Galacturonic acid	0.90 (±0.13)	0.54 (±0.09)

The negative control contained only pectin 0.5%

All the sugars were added to the control medium at 0.5% concentration

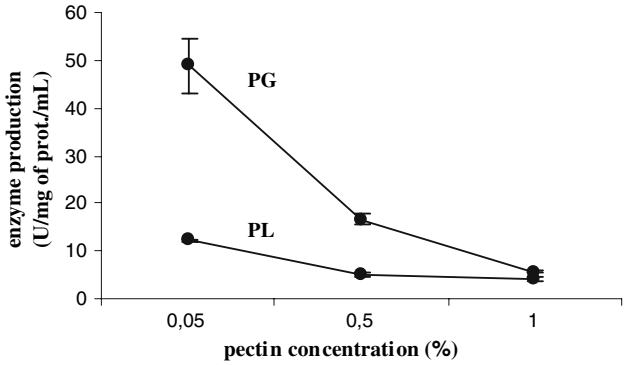

Fig. 4 Effect of pectin concentration on enzymes production. The medium contained pectin as sole carbon source. Error bars represent standard deviation of mean of three replicas

Table 2 Effect of various nitrogen sources on the yield of pectinolytic enzymes

	Enzyme production (U/mg of protein/ml of supernatant)	
Nitrogen compounds	PL (*Bacillus* sp. BS-66)	PG (*Bacillus* sp. BS-6)
Negative control	0.15 (±0.02)	1.58 (±0.20)
Ammonium nitrate	2.74 (±0.30)	1.27 (±0.41)
Meat peptones	1.66 (±0.46)	0.93 (±0.12)
Leucine	1.94 (±0.14)	0.89 (±0.09)
Tryptophan	0.93 (±0.13)	0.47 (±0.05)
Arginine	2.91 (±0.03)	0.33 (±0.05)
Glutamine	2.13 (±0.04)	0.21 (±0.07)
Cysteine	2.48 (±0.12)	1.06 (±0.27)
Yeasts extract	0.89 (±0.09)	0.43 (±0.13)

The negative control contained no nitrogen compound

All the nitrogen sources were added to the control medium at 0.28% concentration

Maximum production of pectinolytic enzymes occurred between 30 and 45°C and above pH 4.0 (Fig. 5). In anaerobic conditions, *Bacillus* sp. BS-66 failed to synthesize PL (Table 4).

PG production

To analyse the conditions of enzyme production, *Bacillus* sp. BS-6 among PG producers was randomly chosen as model. PG production was supported by almost all the carbon sources. A slight increase was noticed with glucose, cellobiose and ribose, while a very significant decrease was observed with sucrose (Table 1). A high production of PG was related to a low concentration of pectin (Fig. 4). The production of PG was more promoted in conditions of nitrogen starvation than when a nitrogen compound was present in the medium (Table 2). However, the level of PG

Table 3 Effect of metal ions on the production of pectinolytic enzymes

Metal ions	Enzyme production (U/mg of protein/ml of supernatant)	
	PL (*Bacillus* sp. BS-66)	PG (*Bacillus* sp. BS-6)
Negative control	4.77 (±0.13)	1.81 (±0.07)
Iron.	16.27 (±1.63)	5.98 (±0.32)
Manganese	1.44 (±0.03)	3.14 (±0.47)
Zinc	0.10 (±0.00)	4.87 (±0.19)
Copper	5.32 (±0.85)	5.01 (±0.47)
Magnesium	1.79 (±0.21)	4.41 (±0.38)

The ions were added at 0.01% concentration to the incubation medium

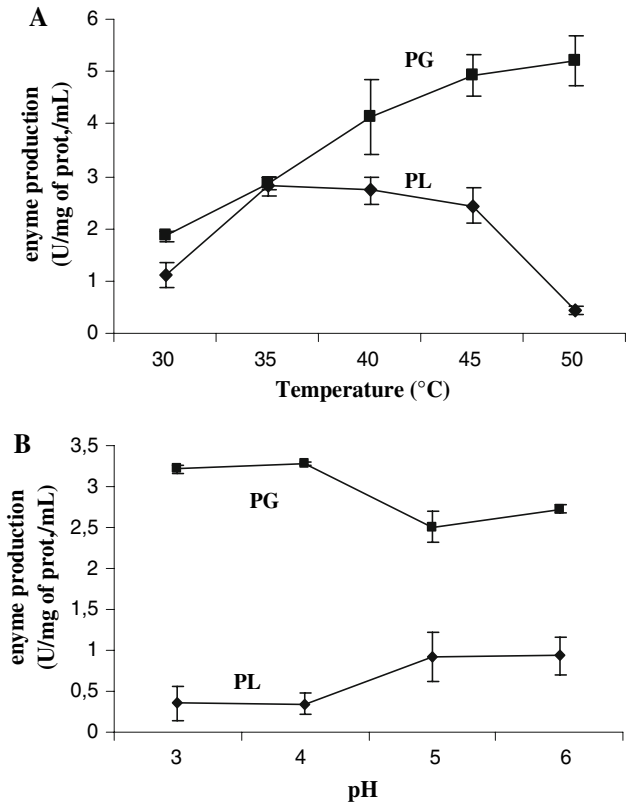

Fig. 5 Effect of pH and temperature of incubation medium on enzymes production

Table 4 Effect of oxygen availability on the production of pectinolytic enzymes

Oxygen availability	Enzyme production (U/mg of protein/ml of supernatant)	
	PL (*Bacillus* sp. BS-66)	PG (*Bacillus* sp. BS-6)
Aerobiosis	8.68 (±0.08)	2.42 (±0.42)
Anaerobiosis	0.16 (±0.01)	3.09 (±0.24)

produced was relatively high with cysteine, ammonium nitrate and meat peptone. A lack of cations in the incubation medium had a negative effect on the PG production (Table 3). Of the cations tested, iron displayed the most inducible capacity, increasing about three-fold the yield of PG in comparison to the negative control. The other metal ions also stimulated enzyme production, but to a lower extent than that observed with iron. When strains were incubated at temperatures lower than 35°C, the amount of PG remained relatively low (Fig. 5). Above 35°C, the yield of this enzyme was heightened to reach a maximum at 50°C. Within the pH 3.0–6.0 range, the amount of PG fluctuated slightly, only from 2.50 to 3.27 U/mg of protein/ml, but a more acidic pH seemed to have a beneficial effect. Oxygen availability did not have any effect on PG synthesis (Table 4).

Lactate and acetate lowered the production of the both enzymes (PG and PL). The effect of lactate was less emphasized than that of acetate, which weakened greatly the PG yield and completely inhibited PL synthesis (Table 5). However ethanol had no effect on the yield of these enzymes.

Discussion

Ninety-eight *Bacillus* strains isolated from cocoa fermentation have been screened for pectinolytic activity by a modified method of Soares et al. (1999) and the conditions of production of theses enzymes by bacilli have been analysed.

The modified screening method used here may be more suitable for accurate classification of an important number of strains based on a clear halo diameter. The modification was aimed at inoculation of the solid screening medium with a liquid inoculum. This allows control of the inoculum size and the medium depth, which proved to be factors influencing the clearance zone diameter (Fig. 6).

It is found that a large proportion (91.83%) of the *Bacillus* strains isolated showed pectinolytic activity. This

Table 5 Effect of microbial metabolites on the production of pectinolytic enzymes

Metabolites	(U/mg of protein/ml of supernatant)	
	PL (*Bacillus* sp. BS-66)	PG (*Bacillus* sp. BS-6)
Control	21.98 (±0.64)	8.62 (±1.08)
Ethanol	22.02 (±2.65)	11.38 (±0.65)
Lactate	12.66 (±0.34)	6.11 (±0.31)
Acetate	0.00	3.55 (±0.50)

The control contained only pectin (0.5%). The metabolites were added to the control medium at 0.5%

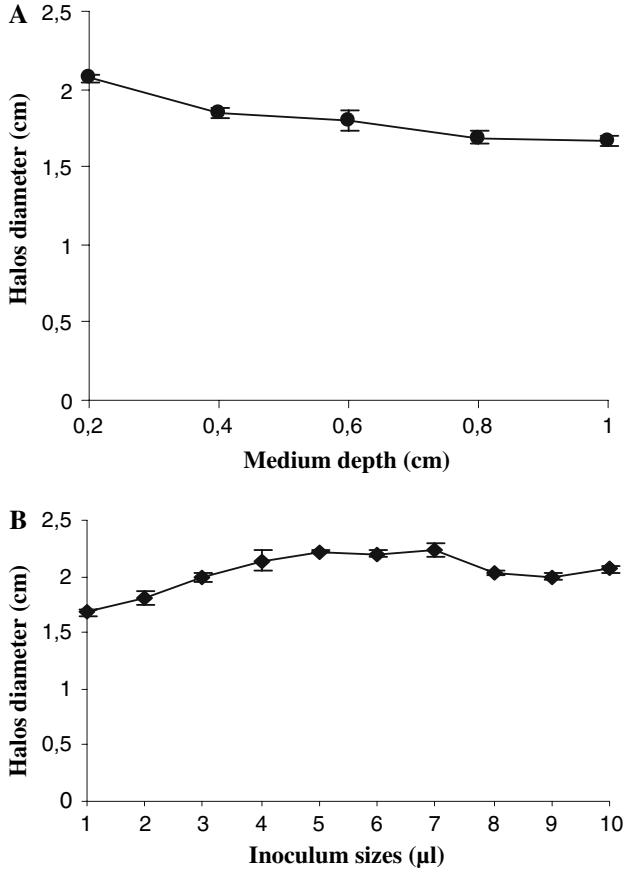

Fig. 6 Halo diameter as a function of inoculum size and depth of screening medium

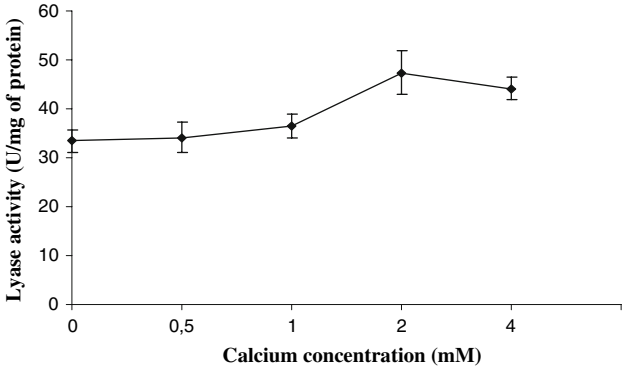

Fig. 7 Effect of calcium on crude enzyme activity

proportion is higher than that of Soares et al. (1999) who reported that 61% of bacilli from soil samples and vegetable wastes were able to produce these enzymes. This should not be surprising since it is assumed that the presence of bacilli in cocoa fermentation is due to contamination of beans from soil at the opening of the pods.

The results show that bacilli strains produced the three types of pectinolytic enzymes, mainly polygalacturonase and pectin lyase and a very low pectin methylesterase activity. Additionally, an increase of lyase activity was observed when $CaCl_2$ was added to the reaction mixture (Fig. 7), suggesting that a calcium-dependant lyase may also be present in the supernatant. Several studies reported the production of calcium-dependant pectate lyase from *Bacillus* (Nasser et al. 1990; Ogawa et al. 2000; Soriano et al. 2006). The lyase system of the studied *Bacillus* sp. from cocoa fermentation could be comprised of pectin lyase and pectate lyase as reported by Soriano et al. (2005) with *Bacillus* sp. BP-7.

To date, no study has been reported on the production of pectin lyase, pectate lyase or pectin methylesterase, by a microbial strain involved in cocoa fermentation; however polygalacturonase produced by yeasts has been previously studied (Schwan et al. 1997).

The pH and temperature changes during cocoa fermentation seem to have less effect on bacilli growth since the growth rate was almost regular in all fermenters. In contrast Schwan et al. (1995) and Schwan (1998) reported a dramatic decline of yeast population after 24 h of fermentation. This emphasizes the important role that *Bacillus* pectinolytic enzymes producer may play in the remaining time of cocoa fermentation.

The analyses of conditions of enzyme production indicate that nitrogen starvation affects strongly the synthesis of PL in *Bacillus* sp. The same effect on pectate lyase was observed in *Erwinia chrysanthemi*, a bacterial strain implicated in the soft rot disease (Hugouvieux et al. 1992). In contrast, condition of nitrogen starvation surprisingly increased PG synthesis. Madhani et al. (1999) have previously reported that transcription of genes can be induced under special condition of nitrogen starvation. However nitrogen depletion is not likely to occur during cocoa fermentation since the proteins in the pulp (Braudeau 1991) and the amount of proteins yielded in the fermenting mass from microflora could be used as nitrogen source. Indeed *Bacillus* sp. was able to utilize a wide range of nitrogenous compounds for pectinolytic enzyme production (Table 2).

Glucose and fructose (at 10% concentration) and to a less extent sucrose (0.7%) are the sugars contained in the pulp (Braudeau 1991). These sugars had no negative effect on PL production. However, a negative effect on PG was observed with sucrose but it could be rapidly hydrolysed into glucose and fructose with the cooperation of the numerous microorganisms present in the fermenting mass. Thus the important amount of readily utilizable carbon sources present in the cocoa pulp could not repress the production of pectinolytic enzymes.

A small amount of pectin (0.05%) was observed to stimulate both PG and PL production in *Bacillus* sp. The same effect was observed by Hsu and Vaughn (1969) in *Aeromonas liquefaciens*. However, the strong decrease of

PL synthesis observed with galacturonic acid at only 0.5% concentration is indicative of a catabolic repression (Table 1). Thus it can be believed that an accumulation of galacturonic acid occurring during fermentation could lead to a limitation of PL synthesis. Nevertheless, even at high concentrations of glucose, sucrose or galacturonic acid (up to 15%), an abolition of catabolic repression is generally observed when strains are cultivated on semi solid state fermentation (SSF) where the microbial cells are grown on a solid surface of materials biodegradable or not (Ramesh and Lonsane 1991a, b; Solís-Pereira et al. 1993). Hence, it is less possible that catabolic repression occurs during cocoa fermentation, since the surface of bean can act as solid support.

Pectin is able to chelate an important quantity of metal ion (Agnieszka 2005). The metal ions studied here seem to have a benefit effect on PG yield, but PL synthesis was severely inhibited by zinc. This inhibition could be explained through the work of De Vries and Visser, (2001) who reported that the protein CreA which mediates the major system responsible for carbon catabolic repression in *Aspergillus* is a zinc finger protein. This suggests a probable existence of a similar catabolic repression system in *Bacillus*. Based on its probable involvement in the carbon catabolic system, we assume that the negative action of zinc in vitro could not be observed in conditions abolishing catabolic repression. In contrast to zinc, iron is found to activate PL production. A similar effect has been reported by Sauvage et al. (1991) who shown a negative effect of pectate lyase production, by iron restriction conditions in *Erwinia chrysanthemi* 3937.

Lactic acid, acetic acid and ethanol are the most important metabolites formed in the fermenting mass (Schwan 1998). Lactate and acetate were revealed to hinder the production of pectinolytic enzymes. Acetate affected more the production of enzymes than lactate, and PL was more affected than PG. However, the microbial metabolites produced are not constantly present during cocoa fermentation (Schwan 1998) so their effect should be transient.

The pH variation from pH 3 to 6, and oxygen availability proved to have less effect on PG synthesis, but the amount of PL was heightened near neutral pH and totally inhibited in conditions of oxygen limitation. This suggests that PL could be synthesized in the latter stage of fermentation, when the fermenting mass is more aerated and the pH is less acid, while the production of PG could be more suitable at the beginning of the fermentation process, in anaerobiosis conditions. It appears that none of the parameters studied inhibits simultaneously the production of both enzymes. So at least one enzyme could be specifically synthesized at a particular stage of cocoa fermentation.

Conclusion

This study is the first report on *Bacillus* pectinolytic strains involved in cocoa fermentation. It indicates that *Bacillus* sp. is able to produce pectinolytic enzymes under cocoa fermentation conditions. So the degradation of the pulp during cocoa fermentation might not be only due to pectinolytic enzymes produced by yeasts but rather by a combined action of enzymes produced by both yeast and *Bacillus* strains.

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