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Effect of Pectin Lyase Enzyme on Fermentation and Drying of Cocoa (*Theobroma cacao* L.): An Alternative to Improve Raw Material in the Industry of Chocolate*

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Abstract

Cocoa (*Theobroma cacao*), in all its presentations, is consumed all over the world and is one of the main drivers of the economic in several countries. The world's Cocoa tendency is focused on developing special beans. This category is subject to postharvest processes of utmost importance such as the fermentation and dry, which are currently carried out with traditional and poorly effective devices, which need to be improved to obtain a high quality product. The aim of this study was to evaluate the influence of the pectin lyase enzyme (E.C.4.2.2.10) on the postharvest cocoa process. We evaluated the enzyme dosage (1.0% and 0.5%) in fermentation and its effect on the variables temperature, acidity and drying time by convection at 60˚C. The Pectin lyase activity during fermentation does not cause a significant effect on the variables of temperature and acidity; however, the drying process time required to achieve 7.0% moisture was reduced. The enzyme dosage of 1.0% was the best result, the amount of exudate obtained (115 ml) during fermentation and the best degree of fermentation (77% ± 3.8) were increased and further shows a change in porosity facilitating the scale surface and internal moisture diffusion. The drying rate (Nw) expressed in kg_{water}/m^2 * min was determined based on the empirical model of Newton, where the higher speed was obtained during the falling period. In conclusion, enzyme dosage 1% was the best concentration evaluated because weaken grain husk, which allowed an adequate fermentation,

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and subsequent time drying reduction until 10.8 h.

Keywords

***Theobroma cacao* L., Pectin Lyase Activity, Fermentation, Convection Drying, Newton Model**

1. Introduction

World production of cocoa 2014/15 (May) reached approximately four million tons with a deficit of 38,000 tons, which will be increasingly inadequate to respond to a demand that is expected from five million in 2020, due to the increase in the consumption of chocolate, confectionery and raw materials (cocoa powder, butter, cocoa liquor and grain) [1]. The rates of productivity in America are lower than the main producer that is still Africa (74.8%). Thus, in the Latin-American, through government plans and in partnership with industry dedicated to the transformation of the cocoa, some strategies are being implemented to cover the growing demand. For example, renewal and improvement of cocoa crops or establishment of manufacture industry in the producing countries closest to the emerging demand (as in the case of Indonesia to cover the Asian market) [2]. Similarly, there have been other strategies for overcoming difficulties of competitiveness caused by weaknesses in the practices of farming, harvest and postharvest that decrease the yields and quality of the grain. The purpose is to stablish the fine cocoa which has greater value and is produced mainly in most of the countries of Latin America, such as Colombia, Ecuador, Venezuela and Peru, which produce 76% of the fine or flavour cocoa of the world [3] [4].

In postharvest there are various technological proposals, specifically in the stages of fermentation and drying, to obtain the optimum development of major components precursors of flavour (reducing sugars, free amino acids, peptides, and organic acids, among others) to produce fine chocolate [5]. In fermentation, the molecular identification of microbial species [6]-[8] has to elucidate the metabolic mechanisms of the degradation of the mucilage and formation of metabolites. In addition, during the drying stage potential species have been identified (*G. xylinum*, *Clostridium sp*) which can be biological markers [9]. At the same time, the development and implementation of the fermentation with yeast starter (*S. cerevisiae*, *H. uvarum*, *P. kluyveri*, *K. marxianus*) and lactobacillus bacteria (LAB) and Acetobacter (AAB) have generated expectation about the direct use of enzymes with activity pectin lyase to enhance the elimination of the mucilage, a decisive factor in the time of duration and effectiveness of the process [10] [11]. The poligalacturonasas (enzymes depolimerizantes endoPG type or exoPG) and pectin esterase have widely been evaluated by the food industry in extraction processes, clarification, fermentation of coffee and tea, among other applications [12]. The mucilage of coffee and cocoa is characterized by a high degree of methylation; in this context, the enzymes with pectin lyase activity (EC 4.2.2.10) can also generate a favorable impact.

One of the most recent studies about pectin lyase was through the application of yeasts that excrete these enzymes during the fermentation, significantly improving the clearance and degradation of the mucilage [13]. Other effects about this enzyme on the quality of fermented and dried cocoa, and its influence on the drying process have not been reported. The biotechnological applications have focused directly on fermentation but it is also important to integrate the drying process for an optimization of the complete postharvest cycle, in order to improve the traditional method of solar drying. This can show a duration up to 2 weeks, with a high potential for microbial contamination and reduce the productive capacity, especially under changing weather [14] [15].

Thus, this research focuses on an improvement of the cocoa postharvest through the evaluation of the effect of enzymes with pectin lyase activity, applied in different concentrations and two different types of cacao clones during its fermentation in order to reduce the drying time by convection to obtain a competitive product in international markets.

2. Materials and Methods

2.1. Materials

Cobs of cocoa, clones Imperial College Selection - ICS 95 and Trinidad Selection Hybrid-TSH 565 were obtained from the cocoa farm “La Nacional” located at 5˚43'2.74"N in the town Tamesis (Antioquia-Colombia), of the harvest in May of 2015. Trinitario clones were chosen because of their high yield and disease resistance

recommended by the National Federation of Cocoa—FEDECACAO for propagation and supported by previous studies on agronomic performance [16]-[18].

Commercial enzyme ROHAPECT® TPL with pectin lyase activity equivalent to 390 PTF/mg (EC: 4.2.2.10).

The chemical compounds used for the characterization were hexane (PubChem CID: 8103), sulfuric acid (PubChem CID: 1118), sodium hydroxide (PubChem CID: 14798) and acetone (PubChem CID: 180).

2.2. Methods

2.2.1. Bromatology Characterization

The samples (crude and fermented) previously dried and ground were characterized through proximal analysis that includes the percentage of ash (Technical Standard Colombia-NTC 5167, 2011), crude fiber (920,168), fat (2003.06) and total protein (2001.11), according to the standard AOAC Official methods (AOAC, 2005).

2.2.2. Use of Pectin Lyase in the Initial Stage of Fermentation

Enzymatic solutions, from 250 ml to 0.5% (treatment B) and 1.0% (treatment C) were applied each one by spraying in 3 kg of cocoa in crude beans and prepared in sacks of gunnysack covered with banana leaves, according to traditional practice [19] [20]. These dosages had not been reported before for reducing the drying time of cocoa, were defined as follows: 0.5% was recommended by the supplier of the enzyme according to the reference that has been used in the fermentation of coffee [21].

To promote anaerobic conditions in the first 48 h, the sacks were placed into propylene containers completely closed until finishing the anaerobic stage (48 h). Subsequently, each experimental unit was aired manually with a frequency of 24 h until the sixth day (144 h). The enzymatic effect was contrasted with a control (A treatment), performed under the same conditions mentioned above and in the absence of enzyme. The temperature was monitored every 24 hours.

2.2.3. Total Acidity

The evolution of the acidity during fermentation was analyzed by titration, expressed in meq of NaOH * 10 g^{-1}. The total acidity was monitored every 24 hours, with removal of samples by triplicate (obtained at different heights) [22].

2.2.4. Drying

The fermented cocoa beans were arranged on grid trays and put into a convection oven (Memmert UFB model 500) at 60˚C with an air velocity of 0.16 m/s; the beans, with a turning frequency of 3 h. The loss of moisture was assessed every 30 min to reach a moisture content of 7%, value established by the Technical Standard Colombian NTC 1252, which regulates the quality of the cocoa beans, fermented and dried.

The initial moisture content in wet basis (X_{wb}) was determined in conventional oven to 105˚C ± 0.2˚C to a constant weight.

The moisture content ($kg_{water}/kg_{dried\ solids}$) and moisture ratio during the drying process were determined using Equation (1) and Equation (2), respectively [23]:

$$\text{Moisture Content} = \frac{M_t - M_S}{M_S} \tag{1}$$

where $M_{(t)}$ is the mass in the time of study and M_s defined as the mass of dried solids obtained by $M_s = M_{(t)} * (1 - X_{wb})$.

$$\text{Moisture Ratio}(MR) = \frac{X_{(t)} - X_E}{X_0 - X_E} \tag{2}$$

where $X_{(t)}$ is the mass of the wet mass in each time of study, X_E is the equilibrium moisture content and X_0 is the initial moisture.

The drying rate (Equation (3)) was determined by the parameter $N_w(kg_{water}/m^2 \cdot min)$, based on change in the moisture in the time.

$$N_w = \frac{M_s}{A_s} * \left(\frac{d_x}{d_t}\right) \tag{3}$$

The term (d*x*/d*t*) was calculated from the empirical model of Newton (Equation (4)), where the constant drying (*K*) was determined by the slope of the graph of Ln(*MR*) vs Time.

$$MR = e^{-Kt} \rightarrow \frac{X_{(t)}}{d_x} = -k\left(X_0 - X_E\right)e^{-k*t} \tag{4}$$

2.2.5. Fermentation Degree, FD (Cut-Off Test)

One of the indicators of the quality of the cacao beans, at the end of the fermentation and drying, corresponds to the fermentation degree done by cutting lengthwise through the middle of the beans. The analysis includes qualitative features of the cotyledon that define a categorization using a sample size of 100 beans (Equation (5)).

$$\text{Degree fermentation} = \frac{\text{Number of beans well fermented}}{\text{100 beans}} \times 100 \tag{5}$$

For a minimum of 65 beans that are brown or dark reddish brown color (chocolate), with well-defined alveoli-shaped and the average mass of at least 120 g is classified as *Premium* category, between 105 and 119 g as *Ordinary* category. Finally, for a minimum of 60 well-fermented beans with an average mass of 40 g is classified as *Pasilla* according to NTC 1252.

2.2.6. Scanning Electron Microscopy (SEM)

The morphology of the husk from the fermented and dried cocoa beans was analyzed by SEM. Samples were fixed in graphite tape coated with gold (Au) in an equipment Quorum (Q300T D) and examined under a microscope JEOL (JSM7100F), with an acceleration voltage of 15 KV, and magnification range of ×270 to ×3300.

2.2.7. Experimental Design

The experimental units were implemented through a completely randomized design (CRD) (**Table 1**). The Repeated Measures in the Time and Factorial models were applied for the stages of fermentation and drying, respectively; both evaluated by an Analysis of Variance (ANOVA) and the comparison of means were carried out to analyze the time of the treatment by Tukey test ($p < 0.05$).

The model used for the analysis of repeated measures in the time described in Equation (6):

$$\hat{Y} = \widehat{\beta_0} + \widehat{\beta_1} x \tag{6}$$

where $\widehat{\beta_0}$ and $\widehat{\beta_1}$ are the coefficients of the model, $\hat{Y}$ represents the average values of the dependent variables, acidity and temperature in fermentation. The value of "*x*" corresponds to the independent variables of time and enzyme concentration.

The model followed for the evaluation of the effects of treatments during drying is described in Equation (7):

$$X_{ij} = \mu + \tau_i + \mathcal{E}_{ij}, \quad i = 1, 2, \cdots, k; \quad j = 1, 2, \cdots, r \tag{7}$$

where:

X_{ij}: the *j-n*th of *i-n*th treatment,

μ: general mean

τ_i: effect of the *i-n*th treatment $\left(\mu_i - \mu\right)$

$\mathcal{E}_{ij}$: deviation of the *j-n*th repetition of the i-nth treatment regarding the mean μ_i, of i-nth treatment

All analyzes were performed using the statistical software R Studio (Version 0.98.1103 GNU Affero General Public License) [24].

Table 1. Schematic of randomization of the DCA.

Treatment	Enzyme concentration (%)	Treatment	Enzyme concentration (%)	Treatment	Enzyme concentration (%)
B	0.5%	A	Control	C	1.0%
C	1.0%	B	0.5%	A	Control
A	Control	C	1.0%	B	0.5%

3. Results and Discussion

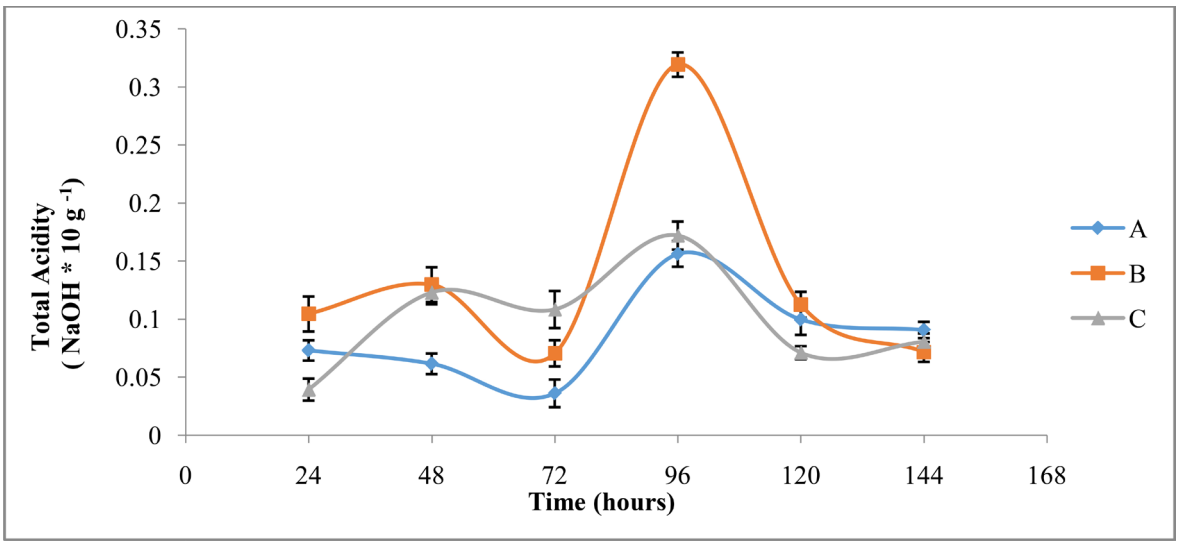
3.1. Fermentation

The bromatology characterization of the cocoa beans without fermenting (% fat 53.13 ± 1.03; % total protein 12.84 ± 0.19; % fiber 2.69 ± 0.09; % ash 9.62 ± 0.14) indicated values that are within the ranges reported for different varieties and country of origin [25] [26]. The fermentation process alters the chemical composition, with a decreasing trend in protein, fat and ash, and an increase in the content of carbohydrates with respect to time of the removal of mucilage [27]. Another effect was observed in the parameter of acidity, as an indicator of the formation of metabolites related to organic acids. In the enzymatic treatments (B, C) and control (A), for 144 hours of fermentation were recorded values of acidity which increased in a range of 0.05 to 0.35 (expressed in NaOH * 10 g^{-1}) (**Figure 1**).

The death of the embryo occurs mainly between the 4th and 5th day (96 hours) when the highest peak of the total acidity were observed for each treatments, consistent with the spread of acetic acid [28]. It is important to outstand the results of total acidity obtained with an enzymatic dose of 0.5% that differs from the control up to 0.16 meq NaOH * 10 g^{-1}, however, the statistical analysis shows that the application of pectin lyase does not generate a significant effect on the acidity of the grain ($p < 0.05$). Guehi *et al.* (2010) reported values of acidity, obtained at the end of the fermentation by different methods (plastic containers, wooden boxes and heaps), higher than the ones found in the present study, which may lead to a better quality by the absence of concentrated acids flavors before the drying process [22]. These results agree with the researches where the use of hybrids of yeast *K. marxianus* increased the activity of the enzyme pectin lyase during the fermentation process [29], producing a positive effect on the sensory quality of cocoa, which is related to the decrease in the acidity.

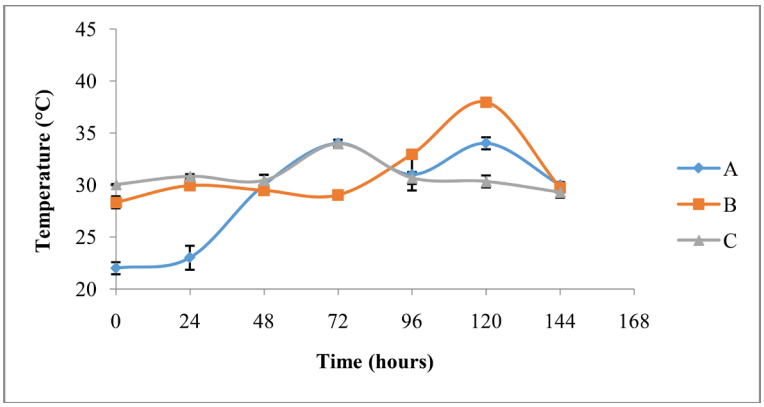
Regarding the variable temperature (**Figure 2**), there is a maximum of 42˚C at 120 hours of fermentation enzyme with a dosage of 0.5%, the increase in temperature is a consequence of the biochemical reactions; in the aerobic phase, oxidation of ethanol to acetic acid corresponds to a process proceeds exothermically. Papalexandratou *et al.* (2013) reported temperatures between 30˚C to 42˚C in conventional fermentation for a load of 500 kg of cocoa, and for 1500 kg a maximum of 50.9˚C. Thus, the amount of cocoa beans in a spontaneous fermentation can be considered as a variable [7].

After fermentation and drying, the analysis of FD and weight (W) of the grain (**Figure 3**) define commercial value related to quality. Some important result was obtained with the treatment C, it exceeded the 65% FD (77% ± 3.8) and 100 g (W) (120% ± 1.2) set for a category *Premium*, it was perceived an acceleration of the biochemical reactions which was evidenced in the physical parameters of the dried cocoa. The use of other commercial enzymes (Ultrazyme) also showed a positive response in the cut-off [30].



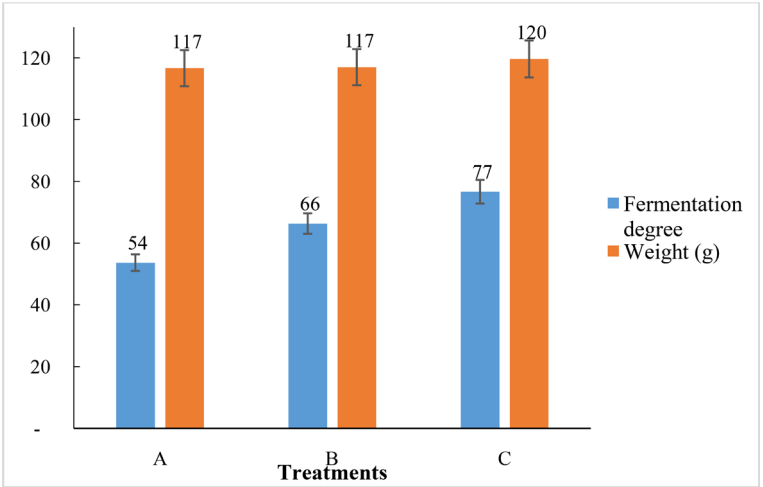
A: Control; B: concentration of pectin lyase enzyme (0.5); C: concentration of pectin lyase enzyme (1.0).

Figure 1. Behavior of the total acidity of the cocoa beans during the fermentation process for the two treatments (B and C) and control (A).



A: Control; B: concentration of pectin lyase enzyme (0.5);
C: concentration of pectin lyase enzyme (1.0).

Figure 2. Behavior of the grain temperature during the fermentation process for the two treatments (B and C) and the control (A).



A: Control; B: concentration of pectin lyase enzyme (0.5);
C: concentration of pectin lyase enzyme (1.0).

Figure 3. Degree of fermentation and average weight of the grain of fermented and dried cacao for the treatments (B and C) and control (A).

Concerning the treatment B, the FD result corresponded to a lower value with respect to the required for the maximum category (66% ± 3.2) possibly because the temperature did not increase in the first 72 h (**Figure 2**) to promote the death of the embryo, necessary to develop the brown color associated to the transformation of different compounds such as anthocyanins [26]. However, there was a significant effect of the pectin lyase for FD with respect to the control treatment A (54% ± 1.2).

The A and B were classified as *Pasilla* but it is important to highlight that both treatments could be classified as *Ordinary* due to the average weight reached (117 ± 1.5 g).

3.3. Drying

The drying time for each of the treatments required between 10 and 15 hours to get a minimum moisture of 7%, which contrasts with the solar drying that can have a duration of 6 and 12 days, depending on environmental conditions. This time is even less than the most recent 55 h solar drying reported in Indonesia [31] [32].

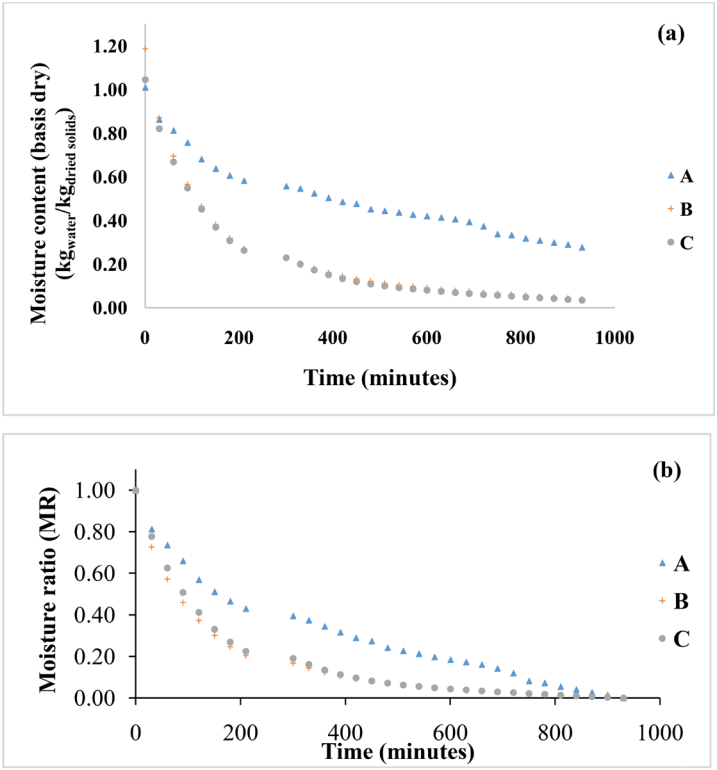
Previous studies of convection drying have proved different operation conditions with the achievement of the decrease in time. Tinoco (2010) reported a drying time of 12 to 15 h carried out at 60˚C with the use of convective system under two conditions. First, dried air removal through dehumidifiers was used, but it had a negative

effect due to superficial burns in grain. The second one was done without removing the moisture from the air, which led to less efficient processes [33]. In the present study, it can be observed (**Figure 4(a)**) that the time for the treatments B and C was less than the processes mention above despite not removing the moisture from the air, possibly due to the action of the pectin lyase.

Figure 4(a) shows the behavior in time of the decrease in the water content expressed in dried basis ($kg_{water}/kg_{dried\ solids}$). It was calculated from the average moisture content, started with the initial moisture of the fermented beans for the three treatments (X_o = 6.1 ± 0.21) up to a final moisture content equivalent to 7% in wet basis (X_F = 0.12 ± 0.01 dry basis) and finally reached the equilibrium moisture content (X_E = 0.067 ± 0.0009).

In **Figure 4(b)** the kinetic shows a reduction in the drying time after application of pectin lyase (B: 680 ± 17.3; C: 650 ± 45.8 min), which denotes a reduction of 190 min at a dosage of 1.0% and 160 min to 0.5%, compared with the control (A: 870 ± 79.4 min). This is because in general, the differential between the moisture content measured in each time and the equilibrium moisture content ($X_{(t)}$-X_E) during the falling period in C was faster than for treatments A and B.

The analysis of variance (ANOVA) allowed to assess the differences between the drying time for the three treatments, which were significant (p = 0.0103). Using the Tukey test it was determined that significant differences existed in A respect to B and C (**Table 2**).



A: Control; B: concentration of pectin lyase enzyme (0.5);
C: concentration of pectin lyase enzyme (1.0).

Figure 4. Evaluation of moisture during the period of drying between 0 and 900 minutes: (a) Moisture content in dry base X_{db}; (b) Moisture Ratio (MR).

Table 2. Comparison of averages for the drying time of treatments, using the Tukey test.

Comparison	Difference	P-value	Low level of confidence	High level of confidence
A vs B	160	0.025243	25.08889	294.91110
A vs C	190	0.011787	55.08894	324.91110
B vs C	30	0.781938	−104.91106	164.91110

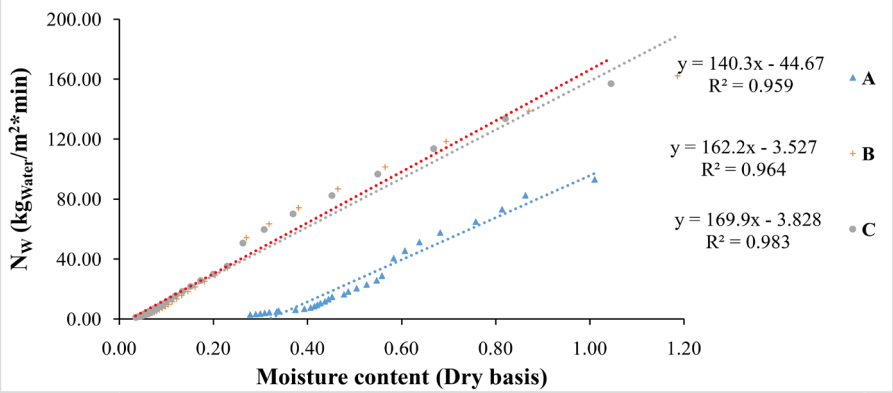
A: Control; B: concentration of pectin lyase enzyme (0.5); C: concentration of pectin lyase enzyme (1.0).

The minimum drying time obtained with the application of the enzyme solution of 1.0% was 10 h, 16.7% less than the convective drying without enzyme reported by Tinoco (2010). On average for B, the drying time was 10.8 h, which did not significantly differ from the application of the enzyme solution to 0.5%, with a drying time on average of 11.3 h. These values were lower than the drying time obtained in previous studies as mentioned above. It is important to note that the initial moisture in the unprocessed cocoa beans (A: 54.3 ± 2.1; B: 57.1 ± 0.7; C: 54.2 ± 5.7) did not present between each treatment, any factor that is considered important in the drying process. Although there were not any relevant differences, the maximum initial moisture value was reached for C (59.9%). However, the drying ended in less time, but in the presence of greater concentration of enzyme.

The determination of the drying rate (N_W) in a range between 0 to 900 min was useful for the understanding of the mentioned behavior about the difference of the time. Besides, Nw may help to make decisions to control the drying periods in order to get the lowest values of volatile acidity, which is one of the most important quality parameters that has to be absent or low as much as possible in the dried cocoa beans [15].

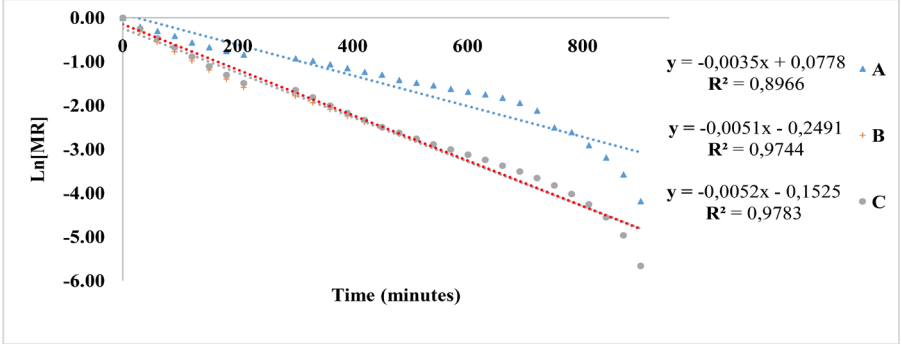
The values of N_w expressed in $kg_{water}/m^2 \cdot min$ were directly related to the decreasing of moisture (**Figure 5**). Since it was a solid material, the constant period of drying was not perceived and only was evaluated from the point of critical moisture balance. The rate for treatments B and C was higher than for A during the entire process, especially since the beginning of the falling period for B (162.0) and C (157.8) compared with treatment A (93.14) under an adjustment with a R^2 between 95.98% and 98.38%. It was related to the amount of free moisture that was not evaporated during the constant period and other volatile compounds as acetic acid. It is expected that the bound water and other non-volatile compounds decrease after getting a N_w of 50 $kg_{water}/m^2 \cdot min$ approximately for B and C.

In the same way, the rate constant (K) obtained from the model, presented a sequence of order equal to the drying rate (N_w) that consisted on A (K: 0.0035 min^{-1}; R^2: 89.66%), B (K: 0.0051 min^{-1}; R^2: 97.44%) and C (K: 0.0052 min^{-1}; R^2: 97.83%) as shown in (**Figure 6**).



A: Control; B: concentration of pectin lyase enzyme (0.5); C: concentration of pectin lyase enzyme (1.0).

Figure 5. Variation in the drying rate (N_W) with respect to the reduction in the moisture content, for the treatments A, B and C.



A: Control; B: concentration of pectin lyase enzyme (0.5); C: concentration of pectin lyase enzyme (1.0).

Figure 6. Rate constant of drying obtained from the model of Newton (slope).

The lower values for the drying time by using the enzyme with pectin lyase activity can be explained by the removal of the mucilage and the change of the porosity of the husk.

A better degradation of the mucilage to B and C was evidenced by the increased amount of exudate during the fermentation (B: 118 ml, C: 155 ml) compared with the control (A: 57 ml). It was the same for the appearance, because the treatments had the least amount of mucilage in the surface (**Figure 7**). Both aspects should allow the free water migrate more easily on the surface. Leal *et al.*, (2008) reported an increase for exudate in the presence of pectin lyase, which impacted the decrease of the fermentation time, without affecting the quality of the grain [29].

The second aspect that can be considered as a facilitator of the evaporation of free moisture mainly in the beginning of the drying and the migration of the volatile compounds by diffusion was the elimination of husks. It was due to degradation throughout fermentation by the action of enzymes with pectin lyase activity on their components present in its structure as the pectin and lignin. The percentage of husks obtained on average in each treatment was for A (23% ± 0.12%), for B (20% ± 0.04%) and for C (12% ± 0.01%), which correspond to a greater presence of enzyme produced a further degradation of husk. This result was related to a lower percentage of fiber for B and C compared with the control at the end of the fermentation (A: 2.32 ± 0.12; B: 1.89 ± 0.17; C: 1.69 ± 0.16) and drying (A 1.59 ± 0.006; B: 1.06 ± 0.005; C: 1.03 ± 0.002).

Figure 8 shows how the surface of the husk changed the morphology as well as the treatment C presented more deformation of the fibers, aspects that were decreasing with treatments B and A, in that order.

The husks from the control presented a more compact fibrous matrix than those in the treatments in presence of enzyme that released the fibers with exposure of a bigger number of folds, represented in spiral. The provision of the non-compact fibers leads to a higher porosity that was proportionally related to the ease of the loss of moisture. Additionally, the final acidity of the dried beans was not affected with the reduced drying time because reached values of 0.135 ± 0.004 meq NaOH * 10 g^{-1} and a pH 5.1 ± 0.1, were between the recommended ranges 0.1 y 0.2 meq NaOH/10 g^{-1} and 4.8 y 5.5, respectively [14] [34]. This could be explained by the diffusion through open and interconnected pores, which may be the subject of future study for a better understanding of the loss of bound water and volatile compounds.

4. Conclusions

The application of the enzyme pectin lyase to 0.5% and 1.0% at the initial point of the fermentation reduced the drying time of the cocoa bean significantly with respect to the control and another drying process. The spray method to apply the pectin lyase during the fermentation was a useful treatment to get the expected action on the

Figure 7. Aspect of the fermented grain: (a) First day with enzyme; (b) Fifth day with enzyme; (c) First day without enzyme; (d) Fifth day without enzyme.

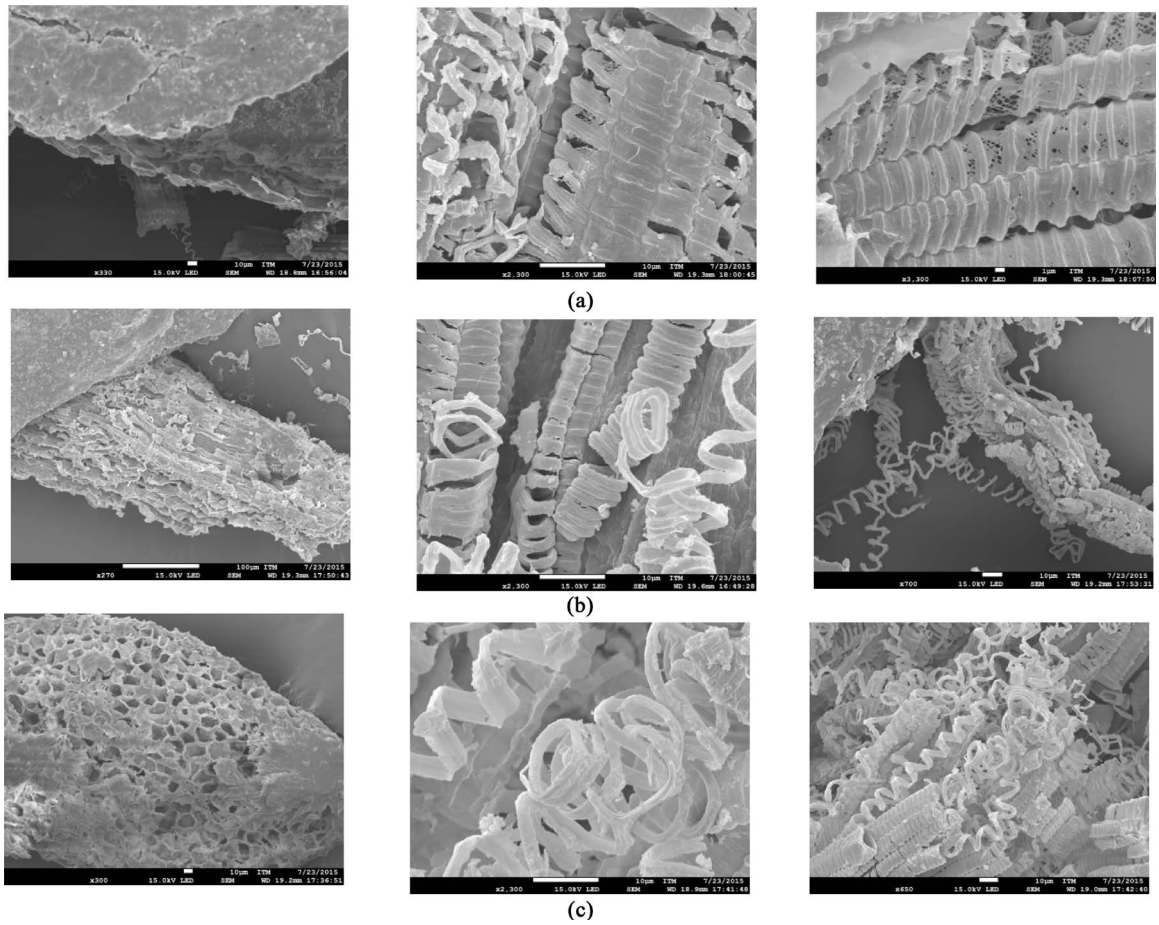


Figure 8. Morphology of the husk of the treatments (a), (b) and (c) as measured by SEM at a scale of breadth of ×270 to ×330, ×650 and ×2300 to ×3300, respectively.

pectin and lignin in order to improve the process of drying additional to the benefit of the conventional application of enzymes to improve the fermentation.

The high quality of the dried cocoa as a raw material obtained in the presence of an alternative to the biotechnology industry of chocolate and its derivatives was reflected with the obtained category *Premium* for 1.0% of the enzyme, based on a greater degree of fermentation and weight in the presence of the enzyme to a moderate concentration, without affecting the application mode of this in a process that is not performed in an aqueous medium.

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