Extraction and determination of invertase and polyphenol oxidase activities during cocoa fermentation

Extração e determinação da atividade de invertase e polifenoloxidase durante fermentação de cacau

DOI:10.34117/bjdv7n8-306

Recebimento dos originais: 07/07/2021
Aceitação para publicação: 02/08/2021

Adriana Barros de Cerqueira e Silva
Doutora em Biologia e Biotecnologia de Microrganismos
Universidade Federal da Bahia
Faculdade de Farmácia, Rua Barão de Jeremoabo, s/n, Ondina, Salvador – BA
E-mail: adrianabarros.cs@hotmail.com

Mariana Barros de Cerqueira e Silva
Mestre em Ciência de Alimentos
Universidade Federal da Bahia
Faculdade de Farmácia, Rua Barão de Jeremoabo, s/n, Ondina, Salvador – BA
E-mail: marianabarros.cs@gmail.com

Paulo Túlio de Souza Silveira
Mestre em Ciência de Alimentos
Universidade Federal da Bahia
Faculdade de Farmácia, Rua Barão de Jeremoabo, s/n, Ondina, Salvador – BA
E-mail: ptssilveira@gmail.com

Eric de Lima Silva Marques
Doutor em Genética e Biologia Molecular
Universidade Estadual de Santa Cruz
Campus Soane Nazaré de Andrade, Rodovia Jorge Amado, km 16, Bairro Salobrinho, Ilhéus – BA
E-mail: marques.ericls@gmail.com

Sérgio Eduardo Soares
Doutor em Ciências dos Alimentos
Universidade Federal da Bahia
Faculdade de Farmácia, Rua Barão de Jeremoabo, s/n, Ondina, Salvador – BA
E-mail: ssoares.ssa@gmail.com

RESUMO
A fermentação é uma das etapas da pós-colheita que mais afeta a qualidade dos produtos obtidos a partir do cacau, pois enzimas oriundas deste processo promovem reações químicas de cura, estabilizando sabor e cor característicos do chocolate. Embora o papel essencial de enzimas endógenas durante essa etapa tenha sido evidenciado há muitos anos, existem ainda poucos estudos sistemáticos abordando a comparação entre diferentes genótipos de cacau, sob diferentes condições de cultivo, com diferentes métodos de fermentação. Além disso, não está ainda elucidado como os processos enzimáticos são

regulados durante a fermentação, que substratos enzimáticos/produtos estão relacionados com o sabor de amêndoas com qualidade superior e quais os fatores limitantes para os processos enzimáticos (disponibilidade de substrato ou enzima, genótipo, condições de cultivo ou processo de fermentação). O presente trabalho visa determinar a atividade das enzimas invertase e polifenoloxidase (PPO) na fermentação dos cultivares de cacau PH 16 e TSH 1188, em cinco tempos distintos da fermentação, baseado nas condições ótimas de atividade previamente estabelecidas no tempo zero (momento imediato antes do início da fermentação). A atividade de ambas as enzimas foi determinada por espectrofotometria para os substratos sacarose e catecol, respectivamente. Os resultados demonstram a diferença e especificidade existente entre os cultivares de cacau, e entre polpa e semente de cada cultivar, além de elucidar a atividade equilibrada das enzimas durante as 156h de fermentação, evidenciando a capacidade das mesmas em se manter ativas ao longo do processo, apesar das intempéries fermentativas. A partir daí, podem ser realizadas intervenções tecnológicas (como controle de pH e temperatura no cocho) que contribuam para melhoria da qualidade da matéria-prima na produção de chocolates monovarietais, que possuem maior valor agregado.

Palavras-chave: Enzimas. Chocolate monovarietal. Precursores de sabor.

ABSTRACT
Fermentation is one of the post-harvest steps that mostly affects the quality of the products obtained from cocoa because enzymes derived from this process promote chemical reactions of healing, stabilizing chocolate's characteristic flavor and color. While endogenous enzymes' essential role during this step has been evident for many years, few systematic studies address the comparison between different cacao genotypes under different growing conditions with different fermentation methods. Moreover, it is unclear how enzymatic processes are regulated during the fermentation, which enzyme substrates/products are related to almonds' flavor with superior quality, and the limiting factors for the enzymatic processes (substrate or enzyme availability, genotype, culture conditions, or fermentation process). This study aims to determine invertase and polyphenol oxidase (PPO) enzymes activities in cultivars PH 16 and TSH 1188 at five different fermentation times, based on optimal conditions of activity previously established at time zero (the immediate moment before the start of fermentation). The activity of both enzymes was determined spectrophotometrically for substrates sucrose and catechol, respectively. The results show the difference and specificity existing between cocoa cultivars and between pulp and seed of each plant variety and elucidate the balanced activity of enzymes during 156h of fermentation, evidencing their ability to remain active throughout the process despite the fermentative elements. Since then, technological interventions (such as pH and temperature control in the trough) can be performed to improve the quality of the raw material in the production of monovarietal chocolates, which have higher added value.

Keywords: Enzymes. Monovarietal chocolate. Flavor precursors.

1 INTRODUCTION

The stages of pre-processing of cocoa (harvest, fermentation, and drying) are essential in ensuring the almonds' quality. Fermentation is one of the post-harvest stages

that most affects the quality of products obtained from cocoa. It is an indispensable activity for developing the precursors of chocolate aroma (LAGUNES-GALVEZ et al., 2007). During this stage, the pulp that surrounds the seeds is metabolized by microorganisms, producing mainly ethanol and acetic acid, which are absorbed by the cotyledons, causing the seed's death and promoting critical physicochemical changes in the flavor formation. Among these, we can highlight the formation of amino acids and free reducing sugars, oxidation of anthocyanins, and complexation of amino acids with phenolic compounds forming quinones, thus reducing bitterness and astringency of almonds (HUANG; BARRINGER, 2010). It is advantageous for

fermentation that microorganisms involved in the process produce specific enzymes, which may have biotechnological applicability not only for fermentative cocoa processes, but also for other industrial processes (SANTOS et al., 2021).

The enzymatic activity in cocoa beans during fermentation has been known and studied since the second half of the 20th century. According to Hansen et al. (1998), it is believed that the enzymes with importance in the formation of the chocolate flavor of cocoa beans are proteases, invertases, and polyphenol oxidase (PPO). Currently, it is known that besides microbial enzymes, endogenous enzymes carry out reactions that lead to the formation of flavor precursors from the cocoa seed itself. That fermentation has the main consequence of lowering the pH, favoring these enzymes' action (MACEDO et al., 2016).

The enzyme invertase acts during this process in the hydrolysis of sucrose in glucose and fructose (reducing sugars), which are essential for the formation of flavor formed by Maillard reaction, which will be enhanced during the roasting stage (HANSEN et al., 1998; LOPEZ; DIMICK, 1991). This enzyme has two isoenzymes: an acid with optimal activity around pH 5.5 and predominant in growing tissues, and a neutral one, with an optimal activity around pH 7.0 and predominantly in fully expanded tissues (HUSSAIN et al., 2008). However, knowledge about neutral and acidic invertases is still limited due to purification difficulties (DU et al., 2013) and low and unstable enzymatic activities (ROITSCH; GONZALEZ, 2004).

Polyphenol oxidase (PPO) is widely distributed in nature and is responsible for catalyzing oxidation reactions of phenolic compounds in the presence of oxygen, whose products polymerize, forming dark-colored compounds (ROBINSON et al., 1991). Its presence and activity during the fermentation and drying of cocoa beans are factors responsible for developing flavor precursors, starting in the oxidative phase of

fermentation and continuing in drying (LIMA et al., 2001). The importance of this enzyme activity is also mentioned implicitly in reducing the bitter and astringent taste of cocoa (REEVES et al., 1988).

Although endogenous enzymes' essential role during cocoa fermentation has been evident for many years, there is still a lack of systematic studies addressing the comparison between different cocoa genotypes, under different cultivation conditions, with different fermentation methods (HANSEN et al., 1998). Besides, the regulation of enzymatic processes during fermentation is still unclear, which enzymatic substrates/products are related to the taste of almonds with superior quality and which are the limiting factors for enzymatic processes, such as substrate or enzyme availability, genotype, cultivation conditions, or fermentation process (MACEDO et al., 2016).

Given the above, the present work aims to determine the invertase and polyphenol oxidase (PPO) activities in cultivars PH 16 and TSH 1188, produced in the south of Bahia. It was made in five different fermentation times, based on the optimal conditions of activity previously established in time zero (the immediate moment before the beginning of fermentation), aiming to provide subsidies for possible technological interventions that can contribute to improving the quality of the raw material in the production of monovarietal chocolates, which have greater added value.

2 MATERIAL AND METHODS

2.1 SAMPLE COLLECTION

Two cocoa cultivars (TSH 1188 and PH 16), produced at Fazenda Lajedo do Ouro, located in the municipality of Ibirataia – BA (S 14°06'15.2" WO 39°38'45.8"), were studied. The cultivar TSH1188 (Trinidad Selected Hybrids - Trinitario hybrid) is originally from Trinidad and Tobago and shows resistance to witches'-broom disease (WBD) and excellent productivity; whereas the cultivar PH 16 (Hybrid Forastero – the result of the crossing of the Forastero from Alto Amazonas with Trinitario) was identified in 1996 in a hybrid cocoa population of Fazenda Porto Hibrido, in the municipality of São José da Vitória - BA.

The samples were collected (approximately 500g) every 12 hours during fermentation until the end of the process and frozen at -18ºC to interrupt the enzymatic activity. During the fermentation, temperature measurements (Digital Thermometer MINIPA, model MT-450) and pH (PHtek Digital Portable pH Meter) of the mass (AOAC, 2000) were performed.

2.2 FERMENTATION

The fermentation process lasted seven days, with cocoa mass turning every 48 hours aiming at its oxygenation and temperature uniformity. The procedure was carried out in a wooden fermentation box (70x70x75cm), with a capacity to store 400kg of cocoa mass. Each cocoa box has 20 holes (1.27cm/diameter) in the bottom and sides to allow the cocoa honey flow produced by the pulp during fermentation. Banana leaves were used to cover the cocoa mass.

2.3 ENZYMATIC CHARACTERIZATION

The enzyme activity was determined in the pulp and seed of the two cocoa cultivars at five different times during fermentation (12h, 48h, 84h, 120h, and 156h), having as parameters the temperature, pH, and the preferred substrate of the enzyme previously determined (MACEDO, 2014; MACEDO et al., 2016) aiming to identify the stage of the process where the conditions are more favorable for the action of invertase and PPO.

2.4 EXTRACTION OF ENZYMES FROM THE PULP

To extract the enzymes from the pulp, 100g of cocoa was used. The pulps were separated from the seeds manually and immersed in a specific buffer solution. The invertase extraction was described by Gomez et al. (1999), where the pulp was immersed in a 50mM pH 7.5 sodium phosphate buffer solution containing 50mM NaCl, 5% glycerol, 5mM manganese sulfate, and 1mM β-mercaptoethanol, in proportion 1:2 (w/v) and homogenized for 30 minutes at 4°C. The homogenate was centrifuged in a refrigerated HITACHI centrifuge, model CR22GIII at 20000.g for 10 min at 0°C.

The extraction of PPO was performed as described by Lima et al. (2001), where the pulp was immersed in a 0.02M potassium phosphate buffer solution (pH 7.5), containing 5% polyethylene glycol and 5mM ascorbic acid, in proportion 1:2 (w/v) and homogenized for 30 minutes at 4°C. The homogenate was centrifuged at 11,000g for 15 minutes at 0°C in the refrigerated centrifuge. The supernatant (extract) of both was then stored at -18ºC for analysis.

2.5 EXTRACTION OF ENZYMES FROM SEED

The seeds removed from the pulp extraction were lyophilized (Lyophilizer Liotop, model L108) and, later, degreased, using petroleum ether as solvent (YUSEP et al., 2002).

The extraction of invertase was carried out as described by Gomez et al. (1999), where the dry and defatted seeds were suspended in buffer solution as described for the pulps. The suspension was then centrifuged at 20,000g for 10 min at 0°C.

PPO extraction was performed as described by Lima et al. (2001), where the dry and defatted seeds were suspended in the same buffer described for the pulps, in 1:10 (w/v) ratio. The suspension was then centrifuged at 11,000g for 15 minutes at 0°C. The supernatant (extract) of both was stored at -18ºC for analysis.

2.6 PARTIAL PURIFICATION OF EXTRACTS

Sufficient ammonium sulfate [(NH4)2SO4] was added to the extracts to provide 80% saturation. The salt was added slowly with gentle stirring at 4ºC and the mixture was centrifuged at 20,000g for 60 minutes at 4°C. The partial invertase purification was performed as described by Deuner et al. (2005), where the precipitate was suspended in 4mL of deionized water. Then, the extract was dialyzed for 24 hours at 4°C with 100mM potassium phosphate buffer at pH 7.0. After dialysis, the extract was precipitated with acetone in the proportion 2:1 (v/v), and its separation was carried out by centrifugation at 11,000g for 15 minutes at 0 °C. The precipitate was resuspended in potassium phosphate buffer pH 6.5 and stored at -18°C for further analysis.

PPO partial purification was carried out as described by Erzengin (2009), where the precipitate was suspended in 0.02M potassium phosphate buffer pH 6.5 and dialyzed against the same buffer in an acetate membrane for 24 hours at 4°C. After dialysis, the protein fraction was precipitated with acetone in the proportion 2:1 (v/v), and its separation was carried out by centrifugation at 11,000g for 15 minutes at 0°C. The precipitate was again suspended in 0.02M potassium phosphate buffer pH 6.5 and stored at -18°C to carry out the other analyzes (PERONE et al., 2007).

2.7 DETERMINATION OF THE ENZYMATIC ACTIVITY OF THE EXTRACTS

The invertase activity was determined according to Nascimento et al. (1998) by spectrophotometry (Biochrom, Model Libra S50), using sucrose as substrate at the concentrations, pH, and temperature conditions established by Macedo (2014) for each cultivar. The reaction mixture (4.0mL) consisted of 0.5mL of the enzyme extract, 1.0mL of sucrose in different concentrations, and 2.5mL of buffer solution at the reaction pH indicated for each isoenzyme, using 0.2M potassium acetate buffer, pH 4.5, for acid invertase and 0.2M potassium phosphate buffer, pH 7.5, for neutral invertase. The

reaction medium was incubated in an incubator for 30 minutes, and the reaction was stopped by heating to 100°C in a water bath. For the cultivar PH 16, the reaction medium presented the following conditions: pH 4.5, sucrose substrate 0.2M and incubation at 33ºC for acid invertase in the pulp; pH 4.5, sucrose substrate 0.4M and incubation at 28ºC for acid invertase in the seed; pH 6.5, sucrose substrate 0.3M and incubation at 52ºC for neutral invertase in the pulp and pH 7.5, sucrose substrate 0.3M and incubation at 50ºC for neutral invertase in the seed. For the cultivar TSH 1188, the conditions were: pH 4.5, sucrose substrate 0.2M and incubation at 31ºC for acid invertase in the pulp; pH 4.5, sucrose substrate 0.4M and incubation at 27ºC for acid invertase in the seed, and pH 7.5, sucrose substrate 0.3M and incubation at 50ºC for neutral invertase in the pulp and seed. The enzymatic activities were evaluated by reducing sugars according to the protocol for determining reducing sugars using the Somogyi-Nelson method (NELSON, 1960). One unit of the enzyme was defined as the amount of enzyme capable of releasing 1 mg of reducing sugar per mg of protein per hour (mg glucose.mg protein^{-1}.h^{-1}).

PPO activity was determined, according to Macedo et al. (2016), by spectrophotometry (Biochrom, Model Libra S50), by measuring the increase in absorbance at 420nm for the catechol substrate. The reaction mixture consisted of 2.5mL of 0.2M sodium phosphate buffer solution (pH 7.0), 0.3mL of 0.1M catechol solution, and 0.2mL of the extract containing the enzyme, reaching a final volume of 3mL. The blank sample had only 0.3mL of the substrate solution and 2.7mL of 0.2M sodium phosphate buffer solution (pH 7.0). For the PH 16 cultivar, the reaction medium had the following conditions: pH 6.5 and 27ºC for pulp; pH 5.8 and 30ºC for seed. For the cultivar TSH 1188, the pH was maintained at 6.6 for the pulp and 6.0 for the seed; the temperature remained constant at 25ºC in both. The linear portion of the activity curve was used to express the activity of the enzyme. A unit of enzyme activity (EU) was defined as the amount of enzyme that causes an increase in absorbance of 0.001.mL^{-1}.min^{-1} (ERZENGIN, 2009).

2.8 DETERMINATION OF PROTEIN CONTENT OF EXTRACTS

For the specific enzymatic activity, the protein content was determined by the method of Lowry et al. (1951).

2.9 STATISTICAL ANALYSIS

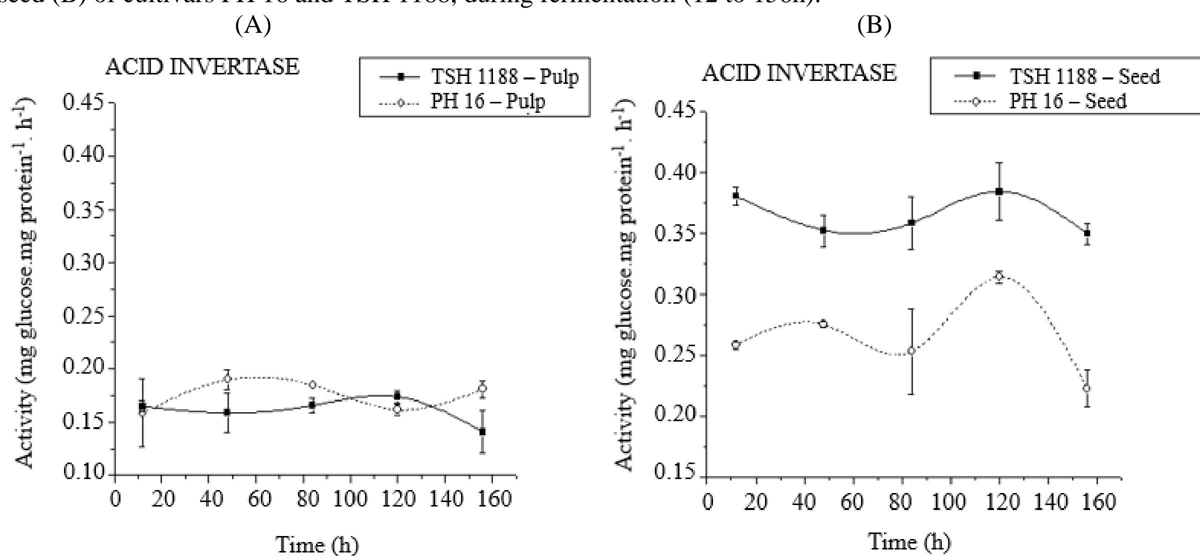
The analyzes were performed six-fold, and the standard deviation of the data was determined.

3 RESULTS AND DISCUSSION

The invertase enzyme acts on sucrose, promoting hydrolysis into glucose and fructose (reducing sugars) during the fermentation process. The participation of this enzyme in the Maillard reaction, which is mainly responsible for forming the desirable taste of chocolate, is crucial (PEZOA-GARCÍA, 1989). In the initial stage, this reaction involves the condensation of carbonyl groups of reducing sugars with amino groups originating, mainly from free amino acids, followed by the degradation of the originated products, forming several oxygenated compounds (ROSLI et al., 1996). These sugars are important precursors of chocolate flavor, and their performance will be enhanced in the later stages of cocoa processing (HUANG; BARRINGER, 2010).

Figure 01 clarifies the activity of acid invertase in the pulp and seed of the two cultivars studied for the sucrose substrate, in the concentrations proposed by Macedo (2014) as being ideal. It is observed that, in general, the isoenzyme, as mentioned earlier, maintained a constant activity throughout the 156h of fermentation, demonstrating that it can remain active throughout the process, despite the variations in pH and temperature that occurred in the fermentation box.

Figure 01 – Acid invertase activity for sucrose substrate 0.2M in pulp (A) and sucrose substrate 0.4M in seed (B) of cultivars PH 16 and TSH 1188, during fermentation (12 to 156h).

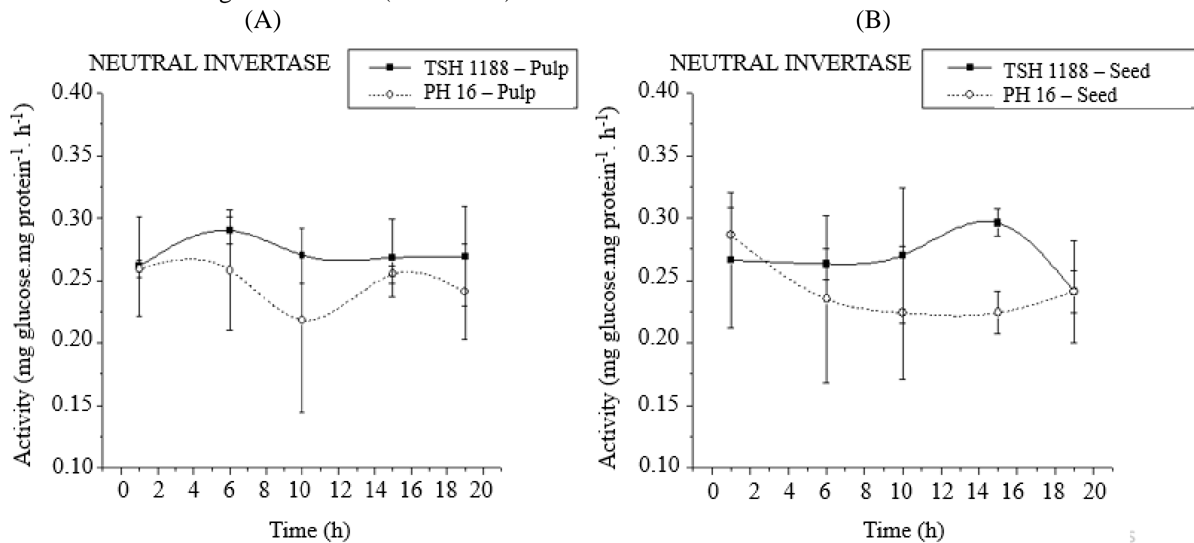


In Figure 01-A, a similar acid invertase behavior in the pulp of the two cultivars can be seen. In the seed (Figure 01-B), the cultivar TSH 1188 presents more significant activity concerning PH 16, indicating a greater presence of the enzyme in this cultivar. Also, both cultivars showed greater activity in the seed than in the pulp; consequently, the difference between the pulp activity and the seed of TSH 1188 was more significant than that of the cultivar PH 16. Despite the cocoa pulp being rich in sugars (10-15%) and the high affinity of invertase for sucrose (MACEDO, 2014), the generated organic acids are generated after fermentation penetrates the seeds. Together with the rise in temperature caused by aerobic fermentation, they make the embryo die and acidify the stored tissue. With the death of the embryo, the selective permeability of the membranes is lost, allowing the contact between enzymes and substrates (LOPEZ, 1986; CRUZ et al., 2013). This suggests a migration of these enzymes from the pulp to the seed, along with the loss of pulp during the fermentation process.

Macedo (2014) describes the monitoring of the pH of the pulp and seed of the two cultivars studied during fermentation. In both, the pulp remains acidic until the end of the process. However, the seed tends to become acidic during the process, which favors the acid invertase activity on the substrate present in the tissue. According to the same author, the optimal pH for the action of acid invertase is close to 4.5. Thus, from the data presented, it can be inferred that, under real conditions, acid invertase has its favored action on the pulp in both cultivars during the seven days of fermentation. In the seed, this enzyme's action is favored for the cultivar PH 16 from 80h, and for TSH 1188, from 90h.

Figure 02 shows the neutral invertase activity in the pulp and seed of the two cultivars studied for the 0.3M sucrose substrate. It is observed that, as with the acid invertase, the neutral invertase also maintained a balanced activity throughout the fermentation, reinforcing the enzyme's potential to resist the process conditions and remain active throughout the seven days.

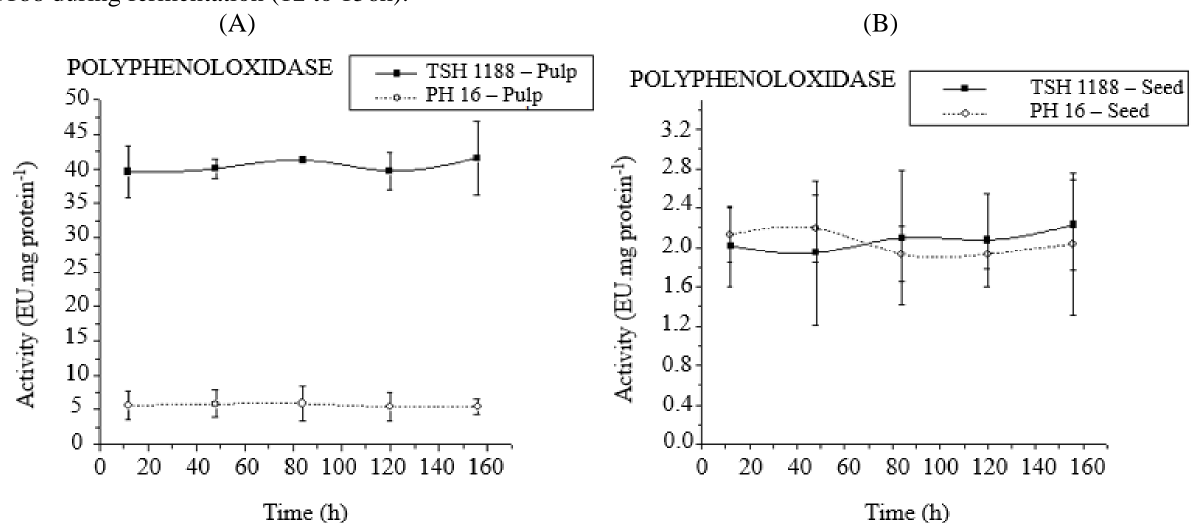
Figure 02 – Neutral invertase activity for the substrate 0.3M sucrose in the pulp (A) and seed (B) of PH 16 and TSH 1188 during fermentation (12 to 156h).



In the case of this isoenzyme, both in the pulp (Figure 02-A) and the seed (Figure 02-B), the activity occurred similarly, both producing between 0.2 and 0.3mg of glucose/mg protein/h. The cultivar TSH 1188 showed higher activity compared to PH 16, corroborating the greater presence of invertase in the cultivar as mentioned earlier. However, neutral invertase (optimum pH values between 6.5 and 7.5) acts outside the pH range throughout the fermentation process. Moreover, it implies that the neutral invertase does not perform these fermentation conditions for the studied cultivars.

Regarding the PPO activity, normal behavior is also observed throughout the fermentation time. This behavior demonstrates that the PPO can remain active despite fermentative conditions (Figure 03).

Figure 03 – PPO activity for the 0.1M catechol substrate in the pulp (A) and seed (B) of PH 16 and TSH 1188 during fermentation (12 to 156h).



In Figure 03-A, there is a significant difference in the enzymatic activity of the two cultivars' pulps. While in cultivar PH 16, the activity remains around 5.0 EU.mg protein^{-1}, in cultivar TSH 1188, this activity remains around 40.0 EU.mg.protein^{-1}, indicating a greater presence of the enzyme in this cultivar. In the seeds (Figure 04-B), the performance of PPO is less than in the pulps and similar in both cultivars due to remarkably similar activity values; consequently, the difference between the pulp and seed activity of the cultivar TSH 1188 is greater than this same difference in the cultivar PH 16. Such results corroborate those obtained by Macedo et al. (2016), where the activity found for the pulp of the cultivar TSH 1188 was about six times greater than that of the cultivar PH 16. Also, the activity of the seeds of the two cultivars showed a similar and much lower behavior than that of the pulps, remaining in a range between 1.0 and 2.5 UE.mg protein^{-1}.

The affinity of PPO for the substrate depends not only on its origin (the type of vegetable) but also on other factors such as species and variety (OKTAY et al., 1995) and its maturation stage and cultivation conditions (GOMES et al., 2001). Besides, it is known that kinetic parameters, such as Km and Vmax, are directly linked to differences in enzymatic activity, so that the lower the Km value, the greater the enzyme's affinity for the substrate (LINEWEVER; BURK, 1934). Also, when studying these cultivars before fermentation, Macedo et al. (2016) observed values of Km and Vmax in the pulp and seed that justifies not only the higher PPO activity in the pulp in relation to the seed but also the difference in activity between cultivars. However, despite the PPO activity in the pulp being higher than in the seed of the two cultivars, considering the natural pH conditions in the fermentation box during fermentation, the action of this enzyme will be favored in the seed, especially in the first three days, where the pH of the medium is closer to its optimum pH of performance, as predicted by the above author.

The high activity of PPO, especially in the pulp of the cultivar TSH 1188, strongly suggests an effect in the content of phenolic compounds, during and after fermentation, due to the intense action of PPO on these compounds at the aerobic phase of fermentation, converting them into quinones (MISNAWI et al., 2002). According to Vamos-Vigyázo (1981), the extent to which these phenolic substrates contribute to enzymatic browning depends on their location and concentration in the substrate, as well as the intensity of the color of macromolecular pigments obtained from quinones, which can lead to changes in sensory characteristics such as color and flavor (MISNAWI et al., 2003). Although

undesirable in most cases, oxidative browning in tea, coffee, cocoa, and prune is desirable (LIMA et al., 2001).

As both enzymes remain active throughout the fermentation process, it is possible to manipulate their performance (aiming to potentiate it) only by controlling factors such as pH and temperature without adding another chemical component. During this control, the specificity of the pulp and seed of each cultivar should also be considered, based on its ideal parameters, providing greater efficiency of the enzyme by prolonging its period of better performance during the fermentation process, which may contribute to the improvement in the characteristics of cocoa beans, and consequently higher quality in chocolate production.

4 CONCLUSION

The study showed the ability of invertase and polyphenol oxidase to remain active during 156h of fermentation in two cultivars widely used in the south of Bahia. Despite the conditions of the fermentation box, it shows typical behavior throughout the process when exposed to optimal conditions of temperature and pH, demonstrating that it is possible to manipulate their performance in real fermentative conditions (aiming to potentiate it) just by controlling the pH and temperature factors, not requiring the addition of another chemical component. Besides, the cultivar TSH 1188 showed behavior that suggests a higher concentration of the studied enzymes concerning the cultivar PH 16.

To improve the production of chocolate with higher value, pH and temperature factors must be controlled during fermentation, considering the specificity of the pulp and seed of each cultivar. It may provide greater efficiency of the enzyme to prolong its period of best performance during the fermentation process. Also, it contributes to the improvement in the characteristics of cocoa beans and consequently greater quality in the chocolate.

ACKNOWLEDGMENT

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/Brasil (CAPES) financial code 001, the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) for funding this study.

REFERENCES

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. **Official Methods of Analysis of AOAC International**, 2000.

CRUZ, J. F. M.; LEITE, P. B.; SOARES, S. E.; BISPO, E. S. Assessment of the fermentative process from different cocoa cultivars produced in Southern Bahia, Brazil. **African Journal of Biotechnology**. v 12, p 5218-5225. 2013.

DEUNER, S. et al. Caracterização parcial da invertase ácida solúvel em tubérculos de batata: energia de ativação e efeito de inibidores. **Revista Brasileira de Agrociência**, Pelotas-RS, v. 11, 45-50, 2005.

DU, L. et al. Characterization of an Invertase with pH Tolerance and Truncation of Its N-Terminal to Shift Optimum Activity toward Neutral pH. **Plos One**. 2013.

ERZENGIN, M. Affinity Purification and Characterization of Polyphenol Oxidase from *Helianthus tuberosus* L. Hacettepe. **Journal of Biology & Chemistry**. 2009.

GOMES, M. R. A.; OLIVEIRA, M. G. DE A.; CARNEIRO, G. E. S.; BARROS, E. G.; MOREIRA, M. A. Propriedades físico-químicas de polifenoloxidase de feijão (*Phaseolusvulgaris L.*). **Ciência e Tecnologia de Alimentos**. Campinas, v.21, p.69-72, 2001.

GOMEZ, M. L. P. A. et al. Metabolismo de carboidratos durante o amadurecimento do mamão (*Caricapapaya*, L. cv. Solo): influência da radiação gama**. Ciência e Tecnologia de Alimentos.** v 19, p 246-52. 1999.

HANSEN, C, E; OLMO, M; BURRI, C. Enzyme Activities in Cocoa Beans During Fermentation. **Journal Science Food Agricultural**. v. 77, p 273 – 281. 1998.

HUANG, Y.; BARRINGER, S. A. Alkylpyrazines and Other Volatiles in Cocoa Liquors at pH 5 to 8, by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). **Journal of Food Science,** v 75, n 1, 2010.

HUSSAIN, A.; RASHID, M.; PERVEEN, R.; ASHRAF, M. Purification, kinetic and thermodynamic characterization of soluble acid invertase from sugarcane. **Plant Physiology et Biochemistry**, 2008.

LAGUNES-GALVEZ, S.; LOISEAU, G.; PAREDES, JL; BAREL, M.; GUIRAUD, J. P. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. **International Journal of Food Microbiology**. v. 114, p. 124-130, 2007.

LIMA, E. D. P. A.; PASTORE, G. M.; LIMA, C. A. A. Purificação da enzima polifenoloxidase (PFO) de polpa de pinha (*Annona squamosa* L.) madura. **Ciência e Tecnologia de Alimentos**, v.21, p. 98-104, 2001.

LINEWEVER H.; BURK, D. The determination of enzyme dissociation constants. **Journal of American Chemical Society.**1934**,** 56, 658-666.

LOPEZ, A. S. **Chemical changes occurring during the processing of cacao. In: Symposium Cacao Biotechnology.** Proceedings. University Park: The Pennsylvania State University, Pennsylvania, p.19-54, 1986.

LOPEZ, A. P.; DIMICK, P. S. Cap. 25: Enzymes involved in cocoa curing In: Food Enzymology Vol.2. **Elsevier Applied Science**. p. 211-236, 1991.

LOWRY, O. H. et al. Measurement with the Folin fenol reagent. **Journal of Biological Chemistry**. 1951, 193, 265- 275.

MACEDO, A. S. L. **Caracterização de enzimas em dois cultivares de cacau *Theobroma cacao* L.** 2014. Dissertação (Mestrado em Ciência de Alimentos). Universidade Federal da Bahia. Faculdade de Farmácia. Salvador – BA, 2014.

MACEDO, A. S. L.; ROCHA, F. S.; RIBEIRO, M. S.; SOARES, S. E.; BISPO, E. S. Characterization of polyphenol oxidase in two cocoa (*Theobroma cacao* L.) cultivars produced in the south of Bahia, Brazil. **Food Science and Technology**. Campinas, 2016.

MISNAWI, J. S.; NAZAMID, S.; JAMILAH, B. Activation of remaining key enzymes in dried under-fermented cocoa beans and its effect on aroma precursor formation**. Food Chemistry**. 2002, 78, 407–417.

MISNAWI, J. S.; JAMILAH, B.; NAZAMID, S. Sensory properties of cocoa liquor as affected by polyphenol concentration and duration of roasting. **Journal of Food Quality and Preference,** v.15, p. 403-409, 2003.

NASCIMENTO, R. et al. Distribuição de amido, açucares solúveis e atividade de invertases em explantes de soja sob várias concentrações de sacarose e diferentes fontes de nitrogênio. **Revista Brasileira de Fisiologia Vegetal.** Viçosa- MG, v. 10, 125-30, 1998.

NELSON, N. A. Photometric adaptation of Somogyi method for determination of glucose. **Journal of Biologic Chemistry**, Bethesda, Md, v. 153, n. 2, p. 375-380, fev. 1960.

OKTAY, M. et al. Polyphenoloxidase from Amasya apple. **Journal of Food Science**.1995, 60, n.3, 494-505.

PERONE, C. A. S. et al. Purificação parcial e caracterização cinética da enzima Polifenoloxidase de banana nanica (*Musa acuminata*). **Revista do Instituto de Ciências da Saúde**. 2007, 25, 239-46.

PEZOA-GARCÍA, N. H. **Contribuition a I'étude d'um capteur por controler em continu procede de torréfaction.** 170p. 1989. These (Docteur) – Université de Technologie de Compiegne, Compiegne, 1989.

REEVES, S. G. et al. Biochemical studies of Cocoa Bean o-diphenol O2 oxidoreductase (catechol oxidase). **Food Chemistry,** Kidlington, 1988, 290-219.

ROBINSON, D. S.; ESKIN, N. A. M. Oxidative Enzymes in Foods. **Elsevier Applied Science,** 1991. Cap 1, p.1-47; Cap 6, p.217-273

ROITSCH, T.; GONZALEZ, M. C. Function and regulation of plant invertases: sweet sensations. **Trends Plant Sci**. 2004, 9, p. 606–613.

ROSLI, W. I. W.; JINAP, S.; RUSSLY, A. R. Effect of roasting time and temperature on volatile components profiles during nib roasting. In: **Proceedings of the 12th**

International Cocoa Research Conference. Salvador, Brasil, Cocoa Producers. P977-991, 1996.

SANTOS, C. M.; SILVA, A. B. C.; MARQUES, E. L. S.; REZENDE, R. P.; PIROVANI, C. P.; FERREIRA, A. C. R.; ANDRADE, L. M.; SANTOS, D. W. C.; DÍAZ, A. M.; SOARES, M. R.; DIAS, J. C. T. Biotechnological starter potential for cocoa fermentation from cabruca systems. **Brazilian Journal of Development.** Curitiba, v.7, n.6, p. 60739-60759 jun. 2021.

VAMOS-VIGYAZO, L. Polyphenol oxidase and peroxidase in fruits and vegetables. **Food Science Nutrition**. v. 49, p. 127, 1981.

YUSEP, I. et al. Influence of carboxypeptidases on free amino acid, peptide and methyl pyrazine contents of under-fermented cocoa beans. **Journal of Science Food Agriculture**. 2002, 82, 1584-92.