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One-factor-at-a-time optimisation of the aqueous extraction of the peroxidase from fresh Amazonian cacao beans

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Abstract

Peroxidase plays an important role in some enzymatic phenomena occurring during the fermentation and drying of cacao beans. Peroxidase reduces the bitterness, astringency, and acidity, and helps develop the characteristic colour and chocolate flavour of cacao beans. The present work thus aimed to optimise five parameters for the aqueous extraction of the peroxidase from fresh Amazonian cacao beans using the one-factor-at-a-time methodology. The following parameters were studied: pH (4 to 10), molarity (50 to 400 mM) of the extraction buffer solution, grinding time (10 to 120 s), homogenisation time after grinding (0 to 90 min), solid-to-liquid ratio between the cacao bean mass, and the extraction buffer solution volume (1:2 to 1:16). The results showed that the recommended pH and molarity of the extraction buffer solution to be 9 and at least 200 mM, respectively. The results also showed that the grinding time to be at least 90 s, and the optimal homogenisation time to be 60 min. The optimal solid-to-liquid ratio must be 1:8. Under these conditions, the activity of the peroxidase increased from 0.01 variation of the absorbance unit per minute (ΔUA/min) to approximately 0.15 ΔUA/min.

Introduction

Peroxidase (POD) (EC. 1.11.1.7) is an enzyme that belongs to the oxidoreductase group. This enzyme catalyses the oxidation of a wide variety of cellular components through a reaction with hydrogen peroxide (H_2O_2) or other organic peroxides as co-substrates. The POD enzyme is present in most microorganisms, animals, and plants (Pandey *et al.,* 2017).

In plants, POD plays an important role in certain physiological processes, especially in the early stages of growth and development, including lignin biosynthesis, cell wall stiffening, hormone regulation, and defence against abiotic and biotic stresses (Francoz *et al.,* 2015; Pandey *et al.,* 2017). In *Theobroma cacao*, POD is involved during the ripening, fermentation, and drying of cacao beans (Sakharov and Ardila, 1999; Rawel *et al.,* 2019). The major POD that functions during these steps is an acidic enzyme with an isoelectric point (pI) of 4.7. Two other basic isoenzymes of the POD enzyme with pIs of 8.6 and 9.0 also function during fermentation (Rawel *et al.,* 2019).

POD in cacao beans is mainly involved in enzymatic browning. This reaction is responsible for the oxidation of mono-, di-, or polyphenols to form *o*-quinones, which subsequently polymerise with other compounds (such as amino acids, peptides, proteins, and sugars) to form coloured polymers (Croguennec, 2016). This reaction is of great importance for reducing the bitterness, astringency, and acidity of cacao beans, as well as for developing the characteristic colour and chocolate flavour of cacao beans (Saltini *et al.,* 2013). From a visual point of view, cacao beans turn from violet at the beginning of fermentation to light brown at the end of fermentation and dark brown at the end of drying (Vámos-Vigyázó and Haard, 1981). This colour change is strongly correlated with the sensorial and organoleptic quality of the cacao beans (Forsyth and Quesnel, 1957).

The present work aimed to contribute to the development of a rapid technique for monitoring the activity of POD during post harvesting operations of cacao beans. Table 1 shows the extraction and quantification of POD from raw materials of *T. cacao* tree carried out in previous works. Most of the previous works focused on the leaves, seedlings, callus, roots, pulp, or embryos of plants. To the best of our knowledge, the only published works related to the activity of POD in fresh cacao beans (or seeds) are those from Ndoumou *et al.* (1995), Sakharov and Ardila (1999), and Li and Sun (1999). In all the works presented in Table 1, POD was recovered by solid-liquid extraction using traditional aqueous extraction (Srinivas *et al.,* 1999). However, as shown in Table 1, no standardised

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Table 1. Results of the works related to the extraction of the POD enzyme from raw materials of the *T. cacao* tree, including the values used for the main parameters of the aqueous extraction.

Reference	Raw materials of the *Theobroma cacao* tree	pH of the extraction buffer solution	Molarity of the extraction buffer solution	Grinding time and technique	Homogenisation time	Solid-to-liquid ratio (w:v)
Atkinson *et al*. (1986)	Leaf	7.8	100 mM	1 min by hand with a pestle and mortar	-	1 cm square / 0.4 mL
Ndoumou *et al*. (1995; 1997); Minyaka *et al*. (2017) *	Pod, **bean**, callus, embryo, root	6.1	50 - 60 mM	? by hand with a pestle and mortar	-	1:3
Okey *et al.* (1997)	Callus	7.8	?	? by hand with a pestle and mortar	-	1:1
Li and Sun (1999)	**Bean**	7.8	100 mM	? ?	-	1:4
Sakharov and Ardila (1999)	**Bean**	7.0	100 mM	? using a bender	1 h	1:10
Resende *et al*. (2002); Nojosa *et al.* (2003) **	Leaf, seedling	6.8	100 mM	? by hand with a pestle and mortar	-	1:50
Ribeiro Júnior *et al.* (2006); Resende *et al*. (2007); Cavalcanti *et al*. (2008); Costa *et al*. (2010) **	Callus, seedling	5.2	50 mM	5 min by hand with a pestle and mortar	-	?
Paz (2010)	Pulp	7.0	50 mM	2 min using a bender	-	1:1
Pirovani *et al*. (2008); Rehem *et al*. (2011); Bertolde *et al*. (2012); Santos *et al*. (2014); Reis *et al*. (2015); Almeida *et al*. (2015); Castro *et al.* (2015); Reis *et al*. (2018) ***	Leaf, root, seedling	6.0	50 mM	1 min sonication	-	1:20
Albores-Flores *et al.* (2018)	Pod	6.8	100 mM	? by hand with a pestle and mortar	-	?

Scopus Base with peroxidase, cocoa, cacao, and seed in the title or the abstract (01/04/2020). * = Department of Biology, University of Yaoundé, Yaoundé, Cameroon; ** = Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, MG, Brazil; *** = Department of Biological Sciences, State University of Santa Cruz, Ilhéus, BA, Brazil; ? = information not given in the paper; - = step not performed in the paper.

methodology has been developed, since the authors started from an already existing method, and adapted it based on their specific matrix without justification.

The main objective of the present work was the optimisation of the aqueous extraction of POD from fresh Amazonian cacao beans using the one-factor-at-a-time (OFAT) methodology. More specifically, the aqueous extraction was optimised based on the following operating parameters: pH of the extraction buffer solution (-), molarity of the extraction buffer solution (mM), grinding time (s), homogenisation time (min), and solid-to-liquid ratio between the cacao bean mass and the extraction buffer solution volume (w:v).

Materials and methods

Raw material

The raw material used in the present work was fresh (i.e., neither fermented nor dried) Amazonian cacao beans obtained from fruits collected in Santa Izabel (PA, Brazil) in July 2017. The fruits were cleaned by brushing under running tap water at ambient temperature. The cacao beans surrounding the pulp were manually removed from the fruits and stored in vacuum-sealed polyethylene packages at -80°C (Panasonic Healthcare model MDF-U33V-PA) until the experiments were performed.

Aqueous extraction of POD

The aqueous extraction was based on the method of Sakharov and Ardila (1999), with some adaptations. First, approximately 30 g of cacao beans were longitudinally and transversely cut using a household knife. Then, they were transferred to a household

blender (Mondial, model NL-22 2 Vel Liq. POWER 2) and ground with an extraction buffer solution. The parameters including the pH and the molarity of the extraction buffer solution, the solid-to-liquid ratio between the cacao bean mass and the extraction buffer solution volume, and the grinding time were optimised. Then, the samples were homogenised by shaking with the aid of a magnetic stirrer table (CAT, model D 79219 Staufen), which was placed inside a BOD incubator (SOLAB, model SL-200/364) and maintained at 4°C. This low temperature was reported by almost all works in the literature related to the extraction of POD from vegetables. The homogenisation time was optimised. The samples were filtered using Whatman filter No. 1. The filtrate was centrifuged at 15,000 *g* for 30 min at 4°C (Hermle Labortechnik GmbH, model Z 326K). The supernatant was recovered and stored at -80°C until the activity of the POD enzyme was quantified.

Experimental plan

The aqueous extraction of the POD enzyme from fresh Amazonian cacao beans was optimised using the OFAT methodology. The following parameters were studied: pH of the extraction buffer solution (-), molarity of the extraction buffer solution (mM), grinding time (s), homogenisation time (min), solid-to-liquid ratio between the cacao bean mass, and the extraction buffer solution volume (w:v). Table 2 presents the sequence of the five steps (no. 1 to 5). The range of values for each parameter was determined based on the literature (Table 1) and preliminary experiments (data not shown). Each experiment was performed in duplicate.

Quantification of POD activity

The POD activity was quantified based on the method of Rogez (2000), with small modifications. The enzyme activity of the diluted enzymatic extract in potassium phosphate buffer pH 5.5 (50 mM) in the presence of *p*-phenylenediamine (2%) and hydrogen peroxide (20 mM) was quantified with a spectrophotometer (Spectro Vision, model T80 +) at 515 nm. The readings were performed in triplicate at 25°C. The oxidation kinetics were measured for 6 min, and the results were expressed as a variation of the absorbance unit per minute (ΔUA/min).

Statistical analysis

The results, expressed as the mean value ± the standard deviation, were calculated based on experimental duplicates and analytical triplicates (n = 6). The results were subjected to one-way analysis of variance (ANOVA) and Tukey's test, with a significance level of 95% ($p < 0.05$), using STATISTICA software version 7.0 (Statsoft Inc., Tulsa/Oklahoma, USA).

Results and discussion

pH of the extraction buffer solution

As shown in Figure 1(A), the activities of POD extracted with extraction buffer solutions at pH values of 4, 5, and 6 were the smallest, and the values were not significantly different from each other (0.08, 0.012 and 0.012 ΔUA/min, respectively). This is probably because the isoelectric point (pI) of POD from fresh Amazonian cacao beans was around these pH values. Sakharov and Ardila (1999) found that the pI of POD from fresh Colombian cacao beans was 4.7. At that pH, the extraction is difficult, since the total charge of the amino acids is zero (Parks, 1967). The POD activity extracted in water was not much higher than the activities at pH values of 4, 5, and 6. This may be because the solubility of POD in aqueous medium is low, making its extraction difficult (Garcıa-Junceda *et al.,* 2004). The POD activity extracted in water was statistically equivalent to that obtained using an extraction buffer solution at pH 7 (0.020 and 0.021 ΔUA/min, respectively). As shown in Figure 1(A), the POD activity extracted from cacao beans was maximal at pH values of 9 and 10, and the values were not significantly different (0.049 and 0.052 ΔUA/min, respectively). This can be attributed to the fact that POD is more

Table 2. Sequence of the steps for the optimisation of the extraction of the POD enzyme from fresh Amazonian cacao beans through an OFAT approach.

No.	pH of the extraction buffer solution	Molarity of the extraction buffer solution (mM)	Grinding time (s)	Homogenisation time (min)	Solid-to-liquid ratio (w:v)
1.	**4, 5, 6, 7, 8, 9, 10**	100	20	0	1:4
2.	Optimal pH	**50, 100, 200, 400**	20	0	1:4
3.	Optimal pH	Optimal molarity	**10, 20, 40, 60, 90, 120**	0	1:4
4.	Optimal pH	Optimal molarity	Optimal time	**0, 15, 30, 45, 60, 90**	1:4
5.	Optimal pH	Optimal molarity	Optimal time	Optimal time	**1:2, 1:4, 1:8, 1:16**

stable in this pH range. However, to avoid working at extreme pH values, pH 9 was selected for the extraction buffer solution for the following assays. It is noteworthy that pH 9 is higher than all the pH values of the extraction buffer solutions used by other authors who extracted POD from raw materials of the *T. cacao* tree (Table 1).

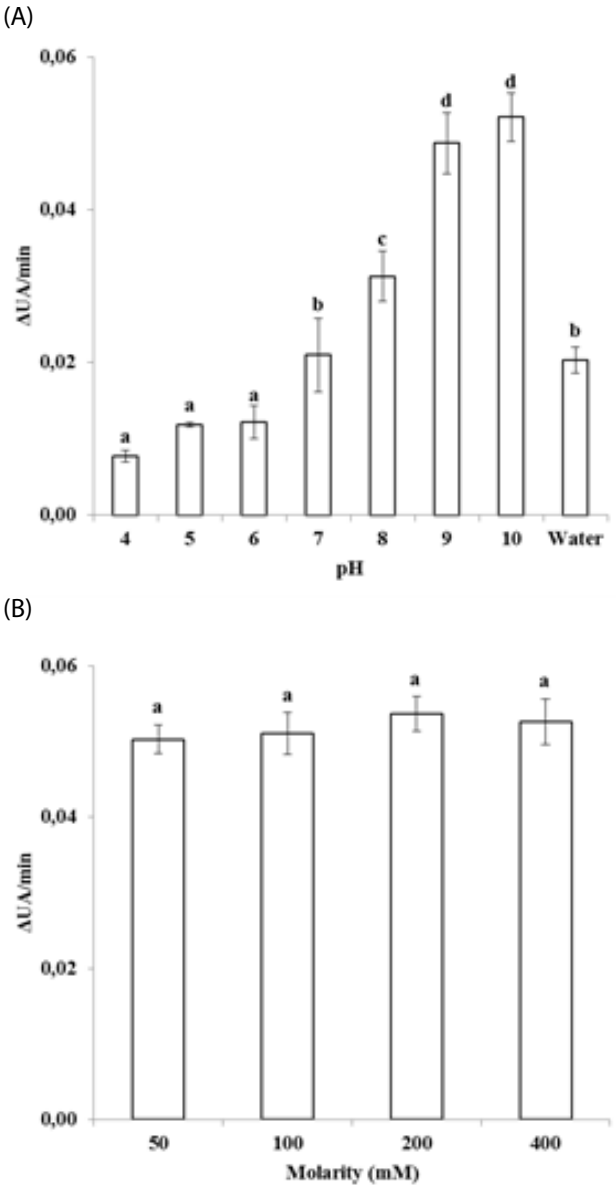

Figure 1. Means ± standard deviations of the activity, expressed in ΔUA/min, of the POD enzyme extracted with extraction buffer solution at different pH values (A), and molarities (B). In (A), the molarity of the extraction buffer solution was 100 mM, the grinding time was 20 s, the homogenisation time was 0 min, and the solid-to-liquid ratio was 1:4. In (B), the pH of the extraction buffer solution was 9, the grinding time was 20 s, the homogenisation time was 0 min, and the solid-to-liquid ratio was 1:4. The same lowercase letters imply that the values are not significantly different based on one-way analysis of variance (ANOVA) and Tukey's test, with a significance level of 95% ($p > 0.05$).

Molarity of the extraction buffer solution

As shown in Figure 1(B), the molarity of the extraction buffer solution (50, 100, 200, and 400 mM) did not significantly affect the activity of POD, with an average value of 0.050 ΔUA/min. Considering that the molar concentration of the extraction buffer solution may influence the efficacy of the enzyme (Oliveira *et al.,* 2010), a parallel experiment was performed to identify the optimal molar concentration for the following experiments. For this purpose, the pH of the extraction buffer solution was measured before (pH = 9) and after the grinding step (20 s). The extractions performed with molarities of 50 and 100 mM showed significantly ($p < 0.05$) higher pH variations (0.64 and 0.34, respectively) than the extractions performed with molarities of 200 and 400 mM (0.10 and 0.07, respectively). Despite this fact, it is interesting that the molarity of the extraction buffer solution used by all works related to the extraction of POD from raw materials of the *T. cacao* tree was 50 or 100 mM (Table 1). The pH values of the extraction buffer solution after the grinding step with solutions at 200 mM and 400 mM were not significantly different ($p > 0.05$). As a too high a molarity may affect the stability of POD, the molar concentration of the extraction buffer solution of 200 mM was selected for subsequent assays.

Grinding time

Figure 2(A) shows that the grinding time had a significant influence on POD activity extracted from cacao beans. The longer the grinding time, the higher the activity of POD. It is interesting to mention that higher grinding times (> 120 s) may be responsible for increasing the temperature of the extraction buffer solution, thus risking denaturation of POD. The experiments performed for 10 and 20 s showed the lowest activities of POD, with values of 0.045 and 0.054 ΔUA/min, respectively. This can be explained by the partial extraction of POD since the grinding times were not sufficient to completely triturate the cacao beans. This could be easily observed visually. The POD activities extracted with grinding for 40 and 60 s were not significantly different (0.084 and 0.081 ΔUA/min, respectively), but were higher than those obtained with lower grinding times. Eventually, when grinding for 90 and 120 s, a significant increase in the POD activity was observed (0.141 and 0.142 ΔUA/min, respectively) as compared to the lower grinding times. This may be explained by smaller particles, higher surface contact, and higher rate of cell disruption of the cacao beans at the end of the grinding step, thus increasing the POD extraction. As these last two grinding times

did not statistically affect the POD activity, the grinding time of 90 s was chosen for the subsequent assays. This parameter is difficult to compare with the literature related to the extraction of POD from raw materials of the *T. cacao* tree since most of the works do not mention either the grinding time or the technique used (by hand with a mortar and pestle or using a blender). The grinding time used in the present work is comparable to that used by Paz (2010), who grinded the pulp for 2 min using a blender (Table 1).

Homogenisation time

The results of the influence of the homogenisation time after grinding on POD activity are presented in Figure 2(B). It can be observed that the results were very similar between different homogenisation times, with values ranging from 0.122 to 0.148 ΔUA/min. This may explain why almost no works related to the extraction of the POD enzyme from raw materials of *T. cacao* tree performed a homogenisation step (Table 1). It is possible to note that no homogenisation (0 min) after grinding, as well as the homogenisation times of 15, 30, and 45 min, did not have a positive impact on the activity, probably due to incomplete solubilisation of POD in the solution (Panadare and Rathod, 2017). Moreover, an excessive homogenisation time (90 min) may allow for the activity of some proteases contained in the cacao beans, since these class-type enzymes can act at pH values on the order of 9 (Fry *et al.,* 1994). Therefore, the homogenisation time of 60 min was the optimal time, as the highest as well as significantly different activity of POD (with 0.148 ΔUA/min) was observed at this time. For this reason, this homogenisation time was selected for the subsequent assays. This homogenisation time was also used by Sakharov and Ardila (1999) who extracted POD from fresh Colombian cacao beans (Table 1).

Solid-to-liquid ratio

The results of POD activity as a function of the solid-to-liquid ratio between the cacao bean mass and the extraction buffer solution volume are presented in Figure 3. The activity in Figure 3(A) is expressed with values ranging from 0.028 to 0.206 Δ UA/min. In particular, the experiment performed with a solid-to-liquid ratio of 1:4 obtained a value of 0.135 ΔUA/min for the activity of the POD enzyme, which is comparable to the value obtained in Figure 2(B) for the same values of the parameters. In Figure 3(B), the activity is expressed by taking into account the concentration of solids in the liquid medium (Δ UA/min/g). The POD activity per gram of cacao

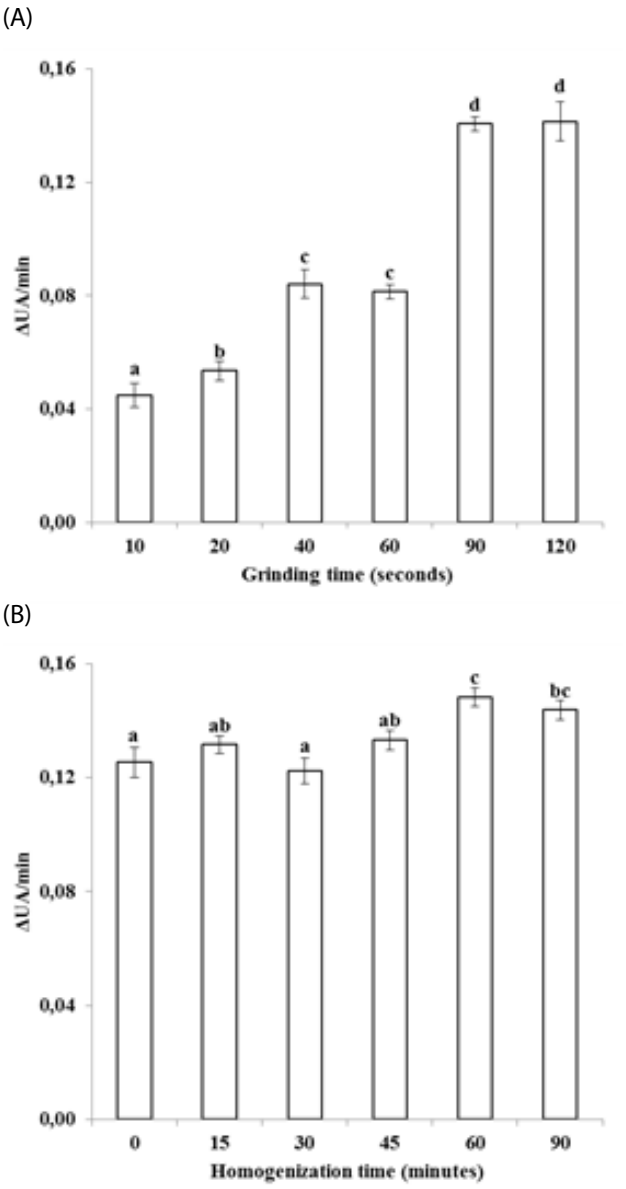

Figure 2. Means ± standard deviations of the activity, expressed in ΔUA/min, of the POD enzyme extracted with different grinding times (A), and different homogenisation times after grinding (B). In (A), the pH of the extraction buffer solution was 9, the molarity of the extraction buffer solution was 200 mM, the homogenisation time was 0 min, and the solid-to-liquid ratio was 1:4. In (B), the pH of the extraction buffer solution was 9, the molarity of the extraction buffer solution was 200 mM, the grinding time was 90 s, and the solid-to-liquid ratio was 1:4. The same lowercase letters imply that the values are not significantly different based on one-way analysis of variance (ANOVA) and Tukey's test, with a significance level of 95% ($p > 0.05$).

beans extracted with solid-to-liquid ratios of 1:2 and 1:4 was low, probably because high bean-to-solution ratios do not allow the complete extraction of POD present in the sample due to solubility saturation (Panadare and Rathod, 2017). On the other hand, POD activity per gram of cacao beans extracted with

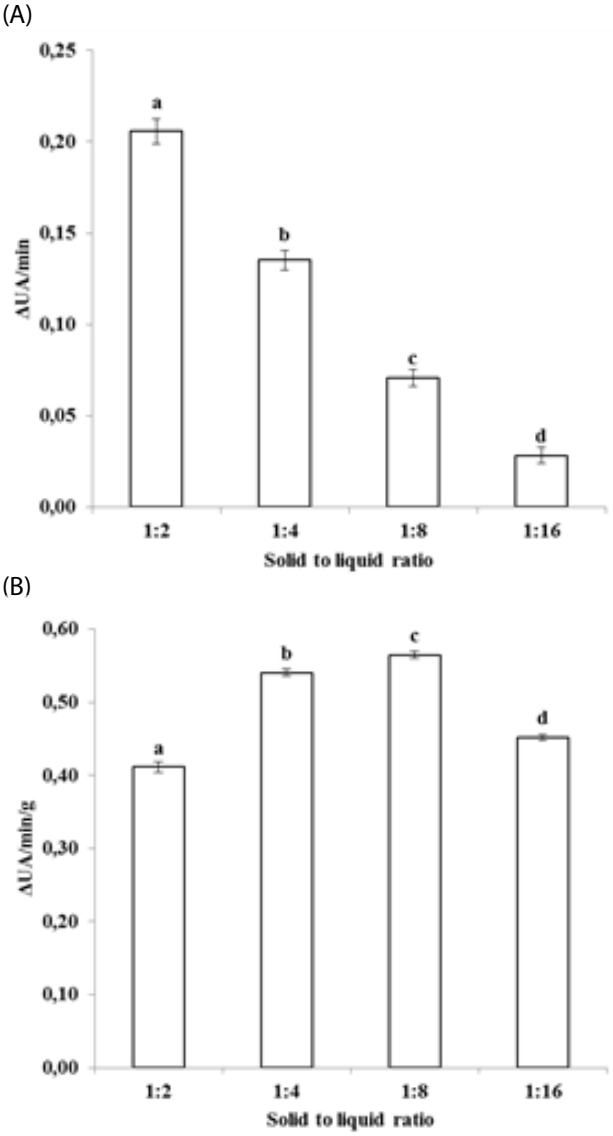

Figure 2. Means ± standard deviations of the activity, expressed in ΔUA/min, of the POD enzyme extracted with different grinding times (A), and different homogenisation times after grinding (B). In (A), the pH of the extraction buffer solution was 9, the molarity of the extraction buffer solution was 200 mM, the homogenisation time was 0 min, and the solid-to-liquid ratio was 1:4. In (B), the pH of the extraction buffer solution was 9, the molarity of the extraction buffer solution was 200 mM, the grinding time was 90 s, and the solid-to-liquid ratio was 1:4. The same lowercase letters imply that the values are not significantly different based on one-way analysis of variance (ANOVA) and Tukey's test, with a significance level of 95% ($p > 0.05$).

a ratio of 1:16 was also low. This may be because an excess of extraction buffer solution was used, and in this way, the unnecessarily dilution of POD in the extract rendered its quantification difficult. Therefore, the optimal solid-to-liquid ratio was 1:8. It is noteworthy that most of the works related to the extraction of POD from raw materials of the *T. cacao* tree used extreme solid-to-liquid ratios (i.e., 1:1 or 1:50) (Table 1). The only work that used a solid-to-liquid ratio comparable to the one identified in the present work was from Sakharov and Ardila (1999) who used a ratio of 1:10 for the extraction of POD from fresh Colombian cacao beans.

Conclusions

In the present work, the optimal parameters of the aqueous extraction of the peroxidase from unfermented Amazonian cacao beans were identified using the OFAT approach. The results suggested using a solid-to-liquid ratio between the cacao bean mass and the extraction buffer solution volume of 1:8 (30 g:240 mL). A set of experiments revealed that the pH of the extraction buffer solution should be 9, and the molarity of the extraction buffer solution must be at least 200 mM. The results also showed that the grinding time should be at least 90 s, followed by a homogenisation time under agitation at 4°C for 60 min. Further research is needed to optimise the aqueous extraction of the POD enzyme from cacao beans collected during postharvest operations, such as fermentation and drying.

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