

Optimization of the recovery yield of the enzymatic aqueous extraction of oil from wet açai decocts using Design of Experiment

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SUMMARY: These last decades, açai oil has been extensively studied for its biological properties and has gained interest from the health industry. It has thus become necessary to develop eco-friendly extraction techniques. The main objective of this study was the use of experimental designs for the maximization of the recovery yield of the enzymatic aqueous extraction process of açai oil from wet decocts. A Simplex-Lattice Mixture Design was employed for the optimization of the proportion of three commercial enzymatic preparations. Subsequently, a Central Composite Design was used to identify the optimal values for total enzymatic concentration (0.5–4.5%) and extraction time (2–12h). The “Response Surface Methodology” (RSM) revealed that the maximum yield ($60.55 \pm 5.98\%$) was obtained using a 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L and Ultrazym AFP-L at a total enzymatic concentration of 2.85% for 10.9 hours. This study concluded that the enzymatic aqueous extraction of açai oil is an efficient and sustainable process.

KEYWORDS: CCD response surface methodology; *Euterpe oleracea*; Mixture design.

RESUMEN: *Optimización del rendimiento de la extracción acuosa enzimática de aceites de açai mediante cocciones utilizando un diseño experimental.* En las últimas décadas, el aceite de açai ha sido ampliamente estudiado por sus propiedades biológicas, resultando interesante para las industrias relacionadas con la salud. Por lo tanto, se ha vuelto necesario desarrollar técnicas de extracción ecológicas. El objetivo principal de este estudio es el uso de diseños experimentales para la maximización del rendimiento en la recuperación del proceso de extracción acuosa enzimática del aceite de açai a partir de cocciones. Se emplea un diseño de mezcla simplex-lattice para la optimización de las proporciones de tres preparaciones enzimáticas comerciales. Posteriormente, se utiliza un Diseño Compuesto Central para identificar los valores óptimos de la concentración enzimática total (0,5–4,5%) y el tiempo de extracción (2–12h). La “Metodología de superficie de respuesta” (RSM) señala que el rendimiento máximo ($60,55 \pm 5,98\%$) se obtiene utilizando una mezcla ternaria 0,49:0,25:0,28 de Celluclast 1,5 L, Viscozyme L y Ultrazym AFP-L a una concentración enzimática total de 2,85% durante 10,9 horas. Este estudio indica que la extracción acuosa enzimática del aceite de açai es un proceso eficiente y sostenible.

PALABRAS CLAVE: CCD metodología de superficie de respuesta; Diseño de mezclas; *Euterpe oleracea*.

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1. INTRODUCTION

The fruit of the species *Euterpe oleracea* is a globose berry, averaging approximately 1 cm in diameter, and 2 g in weight. The pericarp, popularly called pulp, represents only 15% in weight of the fruit, with a thickness of 1-2 mm. During the harvest period, the pulp presents a high concentration of lipids (~30-50% on dry basis, d.b.), proteins (~5-10%, d.b.), and dietary fibers (~20-50%, d.b.) as well as a good source of minerals, phenolic and bioactive compounds (Schauss, 2010; Bichara and Rogez, 2011). The pulp has been widely reported as presenting a high variety of biological activities, mainly anti-inflammatory, antimicrobial, antiprotozoal, antioxidant, antidiabetic and antilipemic (Yamaguchi *et al.*, 2015). More recently, several therapeutic properties have been demonstrated, such as protection against age-related neurodegenerative disorders, cardiovascular diseases, atherosclerosis, control of carcinogenic diseases and global protection of some organs, such as the heart, lung, kidney, and liver (Magalhães *et al.*, 2020).

The presence of the considerable amount of lipids in the pulp of *E. oleracea* fruit, hereafter called açai oil, its chemical composition and biological activities, seems to play an important role in facilitating the absorption of bioactive compounds by the human organism (Skrovankova *et al.*, 2015).

The chemical composition of açai oil is highly similar to that of olive and avocado oil, with more than 70% of unsaturated fatty acids, from which ~60-70% are monounsaturated and ~5-15% are polyunsaturated fatty acids. The most abundant fatty acids are oleic acid (~60-70%), followed by palmitic acid (~15-25%) and linoleic acid (~10-15%) (Pacheco-Palencia *et al.*, 2008; Nascimento *et al.*, 2008; Batista *et al.*, 2016). Furthermore, açai oil contains interesting bioactive compounds, mainly phenolic compounds (Pacheco-Palencia *et al.*, 2008) and tocopherols (Darnet *et al.*, 2011; Lubrano *et al.*, 1994). During the last decade, some works have shown the variety of biological activities and pharmacological applications of açai oil, including antineoplastic, anti-inflammatory, antilipemic, antimicrobial, antinociceptive, cytotoxic, and genotoxic effects (Magalhães *et al.*, 2020). Due to all these properties, açai oil has been proven to be very interesting for food, cosmetic and pharmaceutical industries.

Therefore, it has become necessary to develop and optimize extraction techniques for açai oil. Presently, commercial açai oil is obtained by conventional extraction methods using organic solvents or hydraulic press. Besides being economical and efficient methods, which provide high yields, such techniques are not healthy or environmentally safe. Recently, some works have focused on alternative and more sustainable extraction methods for açai oil, such as supercritical CO₂ extraction (Batista *et al.*, 2016; Silva *et al.*, 2019) or enzymatic aqueous extraction (EAE) (Nascimento *et al.*, 2008), with the aim of obtaining not only a high yield but also a good quality oil. The main disadvantage of the conventional and supercritical CO₂ extraction methods is the need to perform the extraction from a pre-processed and dry product, mainly lyophilized aqueous açai juice, while the EAE process could be performed directly from an “in nature” and wet product (i.e. small pieces of the surrounding pulp of the fruit commonly known as “decocts”).

Commonly, the EAE process of oil from fruit or seed is performed through the succession of three steps: the incubation step (considered as the main one during which the extraction takes place), the solid-liquid separation step (during which the solid residue is separated from the liquid phase) and the liquid-liquid separation step (during which lipid and aqueous phases are separated). During the incubation step, enzymes are specifically selected for increasing the permeability of the different structures of the vegetable cell, mainly the primary and secondary walls, and the oleosomes, thus releasing the oil contained inside it into the aqueous medium (Domínguez *et al.*, 1994; Rosenthal *et al.*, 1996; Ricochon and Muniglia, 2010).

Considering the main composition of the cell wall (i.e. mainly lignocellulose and pectin, and, in minor proportions, glycoprotein and glycolipid), the two most interesting classes of enzymes for partially, or completely breaking down the different molecules are the hydrolases (E.C. 3) and lyases (E.C. 4). In particular, enzymes from subclass E.C. 3.1. (e.g. hemicellulase and pectinesterase) acts on ester bonds, E.C. 3.2. (e.g. cellulase, polygalacturonase and glucosidase) acts on glycosidic bonds, E.C. 3.4. (peptidase-class) acts on peptide bonds, and E.C. 4.2. (e.g. pectin and pectate lyases) acts on the carbon-oxygen bonds (Ricochon and Muniglia, 2010).

These different enzymes can be used simultaneously or successively during the EAE process. Commonly, a mixture of carbohydrases with different activities (e.g. E.C. 3.1., 3.2. and 4.2.) are used during the incubation step. The peptidases (e.g. E.C. 3.4.) can be used in combination with carbohydrases during the incubation step (if the objective is to simultaneously extract oil and protein) or can be added in a second phase, during the liquid-liquid separation step (if the purpose is to de-emulsify the protein-lipid emulsion).

The scientific literature is replete with papers studying the effect of these classes of enzymes on oil yield (and eventually interesting bioactive compounds) extracted by the EAE process from a variety of vegetable raw materials (Yusoff *et al.*, 2015). However, to our knowledge, our research group is the only one to specifically study the EAE process for açai oil for the optimization of its yield.

Numerous unpublished preliminary essays have allowed us to develop a standardized and reproducible EAE process for açai oil. In particular, they indicate that it is more interesting to extract the oil directly from “in nature” wet açai decocts than from any other kind of pre-processed and/or dry raw material derived from açai pulp. This strategy avoids adding water during the juice production, then removing it during drying, as well as significantly limiting the formation of the protein-oil emulsion. Moreover, it has been shown that it is much more efficient to use carbohydrases separately (during the incubation step) and peptidases (during the liquid-liquid separation step). Based on this, our works focused on the maximization of the yield of the oil extracted from açai wet decocts using only carbohydrases during the entire EAE process.

In a previous work, Ferreira *et al.* (2018) studied four commercial carbohydrase enzymatic preparations (EP). They were studied individually and in combination of two, three and four using iso-enzymatic proportion at an individual enzymatic concentration (i.e. liquid enzymatic preparations weight: wet substrate weight, w:w) of 1%. According to the authors, the 1:1:1 ternary mixture of Celluclast 1.5L, Viscozyme L and Ultrazym AFP-L (i.e. at a total enzymatic concentration of 3%) was identified as the optimal one.

The literature shows that other parameters in the EAE process may have a significant impact on the yield of the extracted oil, such as the proportion of the EP, the total enzymatic concentration, the extraction

time, the proportion of water in the mixture, the pH of the medium, the temperature, or the velocity of agitation, amongst others (Yusoff *et al.*, 2015).

In this context, the main objective of our work was the optimization of three parameters – the proportion of the three carbohydrase EP, the total enzymatic concentration and the extraction time – using experimental designs for the maximization of the recovery yield of the enzymatic aqueous extraction process of açai oil from wet decocts using exclusively carbohydrase EP.

2. MATERIALS AND METHODS

2.1. Raw material

E. oleracea fruits were collected in Abaetetuba (Pará State, Brazil) in the middle of the harvest period (GPS coordinates 1°46'42" S–48°51'07" W). Once collected, the fruits were taken to the laboratory, washed under tap water, and softened in water at 40 °C for 1 hour. Then, the softened fruits were pulped by manual friction using sieves. Small pieces of the surrounding pulp of the fruits, with an average size of 0.65 cm, commonly called “decocts”, were obtained. Lastly, the decocts were stored at -20 °C until the EAE experiments were performed.

2.2. Raw material characterization

The dry matter content of the decocts was determined according to the NFTA Method 2.1.4 (AOAC Official Method 935.29 & 945.15) by drying the manually-milled wet decocts in a drying and sterilizing stove (SOLAB SL-100, São Paulo, Brazil) at 105 °C for 3 hours (or until constant weight). The result was expressed as the ratio between the mass of water and the total mass of the wet decocts (% w.b.).

The lipid content of the decocts was determined according to the AOAC Official Method 945.16, by submitting milled dry decocts to Soxhlet extraction (SOLAB SL 145/6, São Paulo, Brazil) using-petroleum ether at 60 °C for 3 hours. The result was expressed as the ratio between the mass of lipid and the total mass of the dry decocts (% d.b.).

2.3. Enzymatic preparations

The three commercial EP (LNF Latino Americana, Rio Grande do Sul, Brazil), were selected according to the conclusions of Ferreira *et al.* (2018):

- a. Celluclast 1.5 L (C): mixture of three synergistic sub-enzymes, namely exo- β -1,4-glucanase (major component), endo- β -1,4-glucanase and β -1,4-glucosidase, 700 EndoGlucanase Units (EGU)/g;
- b. Viscozyme L (V): multi-component carbohydrase containing endo-1,3(4)- β -glucanase (major component), xylanase, cellulase and hemicellulose, 100 Fungal Beta Glucanase Units (FBG)/g;
- c. Ultrazym AFP-L (U): mixture of endopolygalacturonase (major components), endo-pectin lyase, and cellulase, 3000 Pectinase Units (PECTU)/ml.

2.4. EAE process of açai oil

The methodology for the EAE process of açai oil was based on and adapted from Nascimento *et al.* (2008). Each essay was performed using 100 g of thawed wet açai decocts. They were thermally pretreated at 70 °C for 1 min using a domestic food steamer (Walita / Philips Jamie Oliver RI9132/01, São Paulo, Brazil) with the aim denaturing the endogenous enzymes and partially cleave the lignin. Then, the decocts and 240 mL of distilled water at ambient temperature (wet decocts: water $\approx$ 1:2.5 (w:w)) were transferred to a 600 mL beaker sealed with aluminum foil. The three EP were inserted sequentially using a micropipette in the proportion and the total enzymatic concentration according to the experimental designs. A set of triplicate essays was performed without the addition of EP, considered as the negative control.

For the incubation step, the beaker was incubated at 50 °C under orbital agitation of 100 rpm in a shaker (SOLAB SL-222, São Paulo, Brazil) during an extraction time fixed according to the experimental designs. After the incubation, the beaker was cooled in a bath with ice cubes until reaching ambient temperature.

For the solid-liquid separation step, the beaker was left for approximately 24 hours at ambient temperature in order to obtain natural separation of the solid cake (i.e. fibers and high density proteins), the aqueous-proteic phase (i.e. water and low density proteins) and the lipid phase (i.e. oil droplets dispersed on the surface, optionally in protein emulsion). Note that this step may also be realized using a centrifuge lab equipment. The liquid phases (i.e. aqueous-proteic and lipid phases) were then transferred to another smaller beaker.

For the liquid-liquid separation step, about 20 mL of n-hexane (Dinâmica, São Paulo, Brazil) was

added onto the surface with a micropipette in order to favor, on the one hand, the separation of the lipid phase from the aqueous-proteic phase, and on the other hand, the agglomeration of all oil droplets. This strategy was selected as a reference standardized technique at laboratory scale with the aim of focusing only on the effect of the carbohydrase EP (i.e. avoid the use of peptidases) and to allow for the most precise analytical quantification of the extracted oil. Then, the supernatant organic-lipid phase was separated from the aqueous-proteic phase using a micropipette and transferred to a 100-mL previously weighed beaker.

This beaker was incubated (at 100 °C for 15 hours) in a drying and sterilizing stove (SOLAB SL-100, São Paulo, Brazil), until constant oil weight (i.e. complete evaporation of the n-hexane), then stored in a desiccator until reaching ambient temperature. Eventually, the mass of the extracted oil was determined by the gravimetric method, using an Analytical balance (SHIMADZU UniBloc AUY 220, São Paulo, Brazil).

The efficiency of the extraction method, i.e. the oil recovery yield, hereafter called yield (expressed in percentage), was calculated by the ratio between the mass of the oil extracted from 100g of wet decocts by the EAE process, and the mass of the total lipid content in 100 g of wet decocts obtained by the reference Soxhlet method (considered as the positive control).

2.5. Experimental designs

The identification of the optimal values for the parameters was made through two experimental designs: a three-component Simplex-Lattice Mixture Design for the proportion of the three EP and a two-factor Central Composite Design for the total enzymatic concentration and the extraction time. The ranges of values were determined according to the literature (using scopus data base) related to the EAE process for vegetable oils.

2.6. Three-component simplex-lattice mixture design

The effect of the proportion of the three EP on the yield was studied using a three-component augmented Simplex-Lattice Mixture Design. The total enzymatic concentration and the extraction time were fixed at 3% and 4 hours, respectively. Our ma-

trix was carried out with the following 5 coded (and uncoded) levels: 0 (0%), 0.33 (33.3%), 0.5 (50%), 6.6 (66.6%) and 1 (100%). The experimental design presents 15 experimental points, in which 3 are single EP essay treatments (essays 1-3), 9 are two-EP mixture essays (i.e. mid-points, essays 4-12) and 3 are three-EP mixture essays (i.e. central point performed in triplicate, essays 13-15) (see Table 1).

2.7. Two-factor central composite design

The effect of the total enzymatic concentration and the extraction time on the yield was studied using a two-factor Central Composite Design 2^2 with $\alpha = 1.41$. Our matrix was carried out with the following 5 coded levels: -1.41, -1, 0, 1, and 1.41. The to-

tal enzymatic concentrations investigated were: 0.5, 1.08, 2.5, 3.9, and 4.5% (w:w), and the extraction times studied were: 2, 3.5, 7, 10.5 and 12 hours (see Table 2). The central point (2.5% and 7 hours) was performed in triplicate.

2.8. Statistical analysis

The experimental designs and statistical analysis of the data were performed using the STATISTICA software version 7.0 (Statsoft Inc., Oklahoma, USA). The significance of the models and the coefficient estimates of the factors were evaluated by Analysis of Variance (ANOVA) with a significance level of 5% ($\alpha = 0.05$). The optimization was performed using the Response Surface Methodology.

TABLE 1. Three-component augmented Simplex-Lattice Mixture Design: matrix of the proportion (expressed in ratio) of the three EP (enzymatic preparations: C = Celluclast 1.5 L, U = Ultrazym AFP and V = Viscozyme L), results of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) and comparison with Ferreira *et al.* (2018).

Essay n°	Ratio			EAE oil recovery yield (%)	
	C	U	V	Our work	Ferreira <i>et al.</i> (2018) ^s
Negative Control	0	0	0	29.51±5.92 ^s	34.91±11.81
1	1	0	0	42.04	41.52±4.31 *
2	0	1	0	39.14	47.71±11.72 *
3	0	0	1	37.40	46.19±8.31 *
4	0.5	0.5	0	47.08	55.02±13.73 **
5	0.5	0	0.5	45.64	56.20±9.81 **
6	0	0.5	0.5	28.56	46.81±6.15 **
7	0.33	0.66	0	42.74	
8	0.66	0.33	0	50.43	
9	0.33	0	0.66	42.96	
10	0.66	0	0.33	45.56	
11	0	0.33	0.66	36.05	
12	0	66.6	0.33	26.64	
13	0.33	0.33	0.33	51.25	
14	0.33	0.33	0.33	56.81	63.78±3.01
15	0.33	0.33	0.33	49.93	

The essays were performed using a total enzymatic concentration of 3% and an extraction time of 4 hours. The experimental design presents 15 experimental points, in which 3 are single EP essay treatments (essays 1-3), 9 are two-EP mixture essays (i.e. mid points, essays 4-12) and 3 are three-EP mixture essays (i.e. central point performed in triplicate, essays 13-15).

^sThe results are presented as the mean value of triplicate ± standard deviation.

* At a total enzymatic concentration of 1%.

**At a total enzymatic concentration of 2%.

TABLE 2. Two-factor Central Composite Design: matrix of the total enzymatic concentration (expressed in %) and the extraction time (expressed in hours) and results of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %).

Essay n°	Total enzymatic concentration (%)	Extraction time (hours)	EAE oil recovery yield (%)
1	1.08	3.5	45.86
2	1.08	10.5	54.39
3	3.9	3.5	57.61
4	3.9	10.5	59.24
5	0.5	7	48.44
6	4.5	7	48.12
7	2.5	2	50.36
8	2.5	12	60.19
9	2.5	7	58.92
10	2.5	7	56.72
11	2.5	7	57.01

The essays were performed using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L Ultrazym AFP-L, respectively. The experimental design presents 11 experimental points in which the central point (2.5% and 7 hours) was performed in triplicate (i.e. essays 9-11).

3. RESULTS AND DISCUSSION

3.1. Raw material characterization

The decocts presented a dry matter content ranging from $45.14 \pm 0.33\%$ to $46.50 \pm 0.51\%$ w.b. This result is in agreement with those obtained (but not presented) by Ferreira *et al.* (2018) with an average of 50.98% w.b. at the beginning and 45.5% w.b. at the middle of the harvest. No other comparison was possible with the literature because all the authors work from the pre-processed pulp, mainly aqueous juice.

The decocts used throughout our work showed a high variation in the total lipid content, which ranged from $15.20 \pm 0.23\%$ to $24.30 \pm 0.61\%$ d.b., with an average value of $18.82 \pm 3.6\%$ d.b.. This variation in the total lipid content can be justified by the heterogeneity of the vegetable raw material, which may have presented variations in its composition depending on the harvest and the extraction moment itself (Bichara and Rogez, 2011). Our results are slightly lower than those obtained (but not presented) by Ferreira *et al.* (2018), with an average of 28.33% d.b. and 22.45% d.b., at the

beginning and in the middle of the harvest, respectively. These values are significantly lower than those obtained from açai juice by Nascimento *et al.* (2008) with 42.6% d.b., Batista *et al.* (2016) with 45.4% d.b. and Silva *et al.* (2019) with 49-57%. It is worth mentioning that there are more fiber contents in the decocts than the juice, contributing to the reduction in the total lipid content of the decocts compared to the juice.

3.2. Negative and positive control

Considering the mathematical expression of the yield used in our paper, the yield of the positive control (i.e. using the AOAC Official Method 945.16) was 100%, while the yield obtained experimentally of the negative control (i.e. using the EAE process without EP, with an extraction time of 4 hours) was $29.51 \pm 5.92\%$. This result is similar to the one obtained by Ferreira *et al.* (2018) under the same conditions with $34.91 \pm 11.81\%$ (see Table 1). Note that the yields of the negative control are similar to those obtained by other authors who studied the EAE process for oil from different vegetable matrices (see Table 3).

TABLE 3. Comparison of the recovery yield (%) of the oil extracted by the EAE process from different vegetable matrices, using similar values for the total enzymatic concentration and extraction time to ours, and using several enzymatic preparations, including cellulase, hemicellulase and/or pectinase, individually and in combination (using iso-enzymatic proportion).

Reference	Matrix	Enzymatic preparations			Total enzymatic concentration (%)	Extraction time (hours)	Oil recovery yield (%)						
		Cellulase	Hemicell.	Pectinase			Negative Control	1 EP	Cell.	1 EP	Hemicell.	1 EP	Pect.
Our work	Açai decocts	C	V	U	3	4	29	42	37	39	28-47	53	4 EP
Ferreira <i>et al.</i> 2018	Açai decocts	C	V	U	1-3	4	34	41	46	47	46-56	63	
Abdulkarim <i>et al.</i> 2006	Moringa seed	C	-	other	2	36	35	65		56			74 *
Zhang <i>et al.</i> 2007	Rapeseed	other	-	other	2.5	3	48	69		85	75-88	84 **	
Tabatabaei and Diosady, 2013	Yellow mustard flour	C	V	other	3	3	55	67	68	65	72	76	
Mai <i>et al.</i> 2013	Gac fruit	other	-	other	10	2	5-10	12		20	29	40 #	62 #
Dela Cruz <i>et al.</i> 2007 @	Vutalao seed	other			2	4	29	79			97 \$	93 \$	
Silvamanya and Jahim 2015 @	Palm	other	Other	other	0.3	2	53	73	79	71	71-79	90	

Legend: C=Cellulast 1.5L, U=Ultrasym AFP-L, V=Viscozyme L, EP=Enzymatic Preparation. @ use of Simplex-Lattice Mixture Design, * including a protease and a amylase, ** including a glucanase, # including a amylase and/or a protease, \$ including a amylase and/or a xylanase

3.3. Three-component augmented Simplex-Lattice Mixture Design

The matrix of the experimental design and the results of the three-component augmented Simplex-Lattice Mixture Design for the maximization of the yield as a function of the proportion of the three EP is presented in Table 1. One can easily observe a significant increase in these yields, whatever the proportion used, compared to the negative control.

Our experimental results can be directly compared with the ones obtained by Ferreira *et al.* (2018), who studied the effect of these three EP individually and in combination using iso-enzymatic proportion at an individual enzymatic concentration of 1%, on the yield of the oil extracted from açai wet decocts. One can observe that our results, all obtained at an enzymatic concentration of 3%, are slightly lower (see essays 1-3, essays 4-6 and essays 13-15, Table 1). One can observe a variability in our results at the central point (see essays 13-15, Table 1), with an average experimental yield of $52.63 \pm 3.66\%$. Such variability may be explained by the use of biological materials, and the independent repetition of all the essays. Note also that the central point provides the maximum experimental yield.

Model fitting. The fitting of the different order models (i.e. linear, quadratic or cubic) to the experimental results of the yields showed that the cubic was the most appropriate one. In particular, the low global p-value (< 0.0028) indicated that the model was significant, the high R^2 (> 0.90) was satisfactory to validate the significance of the model, the high R^2_{adj} (> 0.83) showed a good relationship between the experimental data and the fitted model and the high lack-of-fit p-value (> 0.6) indicated that the model seemed to accurately fit the experimental data. The significant coefficients (p-value < 0.05) of the factors of the cubic model are those of the linear effects (p-value < 0.0001), followed by the cubic effect (p-value = 0.0026), and the quadratic effect between Ultrazym AFP-L and Viscozyme L (p-value = 0.0415).

Optimal value of the proportion of the three enzymatic preparations. The optimal value of the proportion of the three EP for predicted maximum yield was determined by Response Surface Methodology. In particular, the contoured response sur-

face using the special cubic model clearly showed that the highest yields were achieved with enzymatic mixtures containing more than 25% of Celluclast 1.5L (see red colored region in Figure 1). According to the prediction profiling, the judicious proportion of the three EP which maximized the yield was 45.9% of Celluclast 1.5 L, 25.3% of Viscozyme L and 28.8% of Ultrazym AFP-L (see red vertical line that indicated the optimal value of each EP, in Figure 2). In these conditions, the predicted maximum yield was $54.07 \pm 8.91\%$. Note that this value is comparable to the maximum yield obtained experimentally (see essays 13-15, Table 1).

Understanding of the enzymatic phenomena.

Our results demonstrate that Celluclast 1.5L allowed for obtaining higher yield than using Viscozyme L or Ultrazym AFP-L (see essays 1-3 in Table 1). This can be explained by the fact that endo-enzymes randomly cleave internal bonds of the polymer chain, while exo-enzymes act on the end of the polymer chain. In particular, exo-glucanase (e.g. Celluclast 1.5 L) is essential in the breakdown of crystalline cellulose, and is considered an “input enzyme” for the releasing of oil contained in the vegetable cell (Dominguez *et al.* 1994). However, due to the overlapping of the

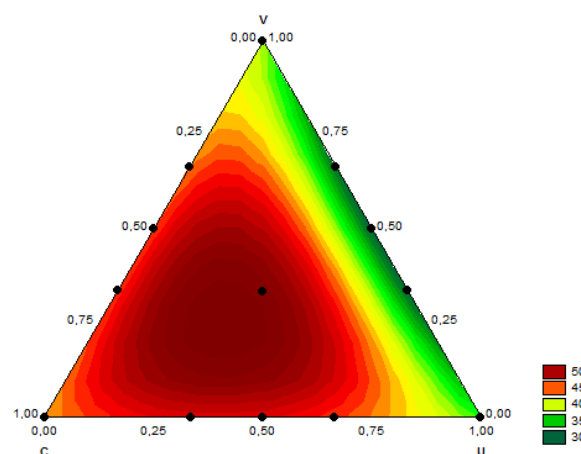


FIGURE 1. Contoured Response Surface of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the ratio of the three EP (enzymatic preparations): C = Celluclast 1.5 L, U = Ultrazym AFP and V = Viscozyme L) using the cubic model for the three-component augmented Simplex-Lattice Mixture Design.

The assays were performed using a total enzymatic concentration of 3% and an extraction time of 4 hours. The experimental design presents 15 experimental points, in which 3 are single EP essay treatments (i.e. vertex of the triangle), 9 are two-EP mixture essays (i.e. on the edges of the triangle) and 3 are three-EP mixture essays (i.e. central point performed in triplicate).

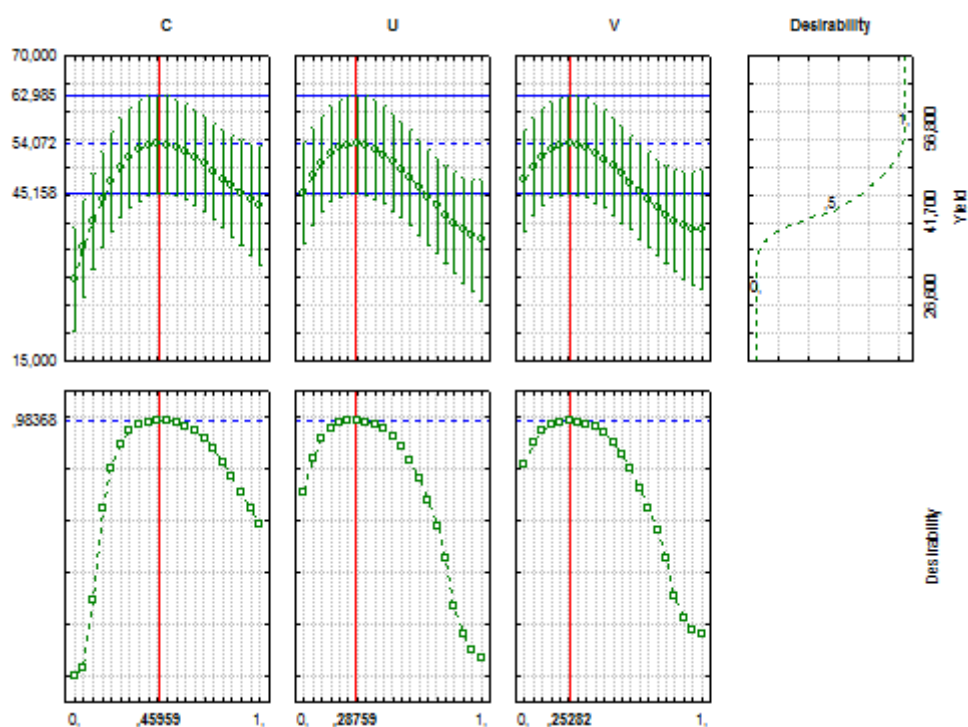


FIGURE 2. Prediction profiling (with prediction intervals) and desirability function of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the ratio of the three EP (enzymatic preparations: C = Celluclast 1.5 L, U = Ultrazym AFP and V = Viscozyme L) using the cubic model of the three-component augmented Simplex-Lattice Mixture Design. The essays were performed using a total enzymatic concentration of 3% and an extraction time of 4 hours.

different polymers of the vegetable cell wall, the accessibility of Celluclast 1.5 L to its specific substrate is intrinsically low. Therefore, enzymatic mixtures containing other class-type enzymes, in particular endo-enzymes, are more competitive. Our results clearly demonstrate that the enzymatic mixtures of two EP containing Celluclast 1.5L with Viscozyme L or Ultrazym AFP-L show yields higher than those obtained with the use of only one EP (see essays 4-5, 7-10 in Table 1, and red color region in Figure 1). This finding can be explained by the fact that these two endo-enzymes easily and randomly act on different polysaccharides of the vegetable cell wall, and in particular generate ends of cellulose chain for the subsequent action of Celluclast 1.5L (Silvamany and Jahim, 2015). On the other hand, as already pointed out by Ferreira *et al.* (2018), the essays carried out without Celluclast 1.5 L are considered non-advantageous as showing yields lower than those obtained with the use of only one EP (see essays 6, 11-12 in Table 1, and green color region in Figure 1).

Comparison with the literature. Some papers have already studied the effect of several EP, includ-

ing cellulase, hemicellulase and/or pectinase, individually and in combination (using iso-enzymatic proportion), on the yield of the oil extracted by the EAE process from different vegetable matrices, using similar values for the enzymatic concentration and extraction time to ours (see e.g. Abdulkarim *et al.*, 2006; Zhang *et al.*, 2007; Tabatabaei and Diosady, 2013; Mai *et al.*, 2013, and Ferreira *et al.*, 2018, Table 3). As observed, the results obtained by these authors are of the same order as those obtained in our work. Most of authors pointed out that the cellulase and the pectinase are among the main enzymes which contribute to increasing the yield. Moreover, most of the papers concluded that a synergetic action of the different EP on the vegetable cell wall is necessary to maximize the release of oil.

However, to our knowledge, the literature presents very few works related to the optimization of enzymatic mixtures for the aqueous extraction of oil by employing a Simplex-Lattice Mixture Design. One can cite the papers of Dela Cruz *et al.* (2007) and Silvamany and Jahim (2015) (see Table 3). More specifically, Dela Cruz *et al.* (2007) ob-

served that ternary mixtures did not provide significantly higher yields than binary mixtures, and concluded that the maximum yield was achieved using a 1:1 binary mixture of xylanase and cellulase (or amylase). Silvamany and Jahim (2015) showed that maximum experimental yield was achieved using a 1:1:1 ternary mixture of cellulase, hemicellulase and pectinase, an a maximum predicted yield with ratio 0.46:0.34:0.2 (celulase : hemicelulase : pectinase). Such an enzymatic proportion is very similar to that obtained in our work.

3.4. Two-factor Central Composite Design

Table 2 shows the matrix of the experimental design and the results of the two-factor Central Composite Design for the maximization of the yield as a function of the total enzymatic concentration and the extraction time, using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L and Ultrazym AFP-L, respectively. Once more, one can observe a variability in the results at the central point (i.e. total enzymatic concentration: 2.5%, extraction time: 7 hours) (see essays 9-11, Table 2), with an experimental average yield of $57.55 \pm 1.19\%$. One can observe that the results obtained for the essays realized with a total enzymatic concentration of 2.5% and extraction times of 2 and 7 hours (see essays 7 and 9-11, Table 2) are coherent with the one predicted by the previous model for a total enzymatic concentration of 3% and an extraction time of 4 hours. Note that the highest experimental result was obtained for essay 8, with 60.19%, performed using a total enzymatic concentration of 2.5% and an extraction time of 12 hours.

Model fitting. The fitting of the different order models (i.e. including linear, quadratic or interaction effects, and their combination) to the experimental results of the yields showed that the simpler second order models (i.e. that combine the linear & quadratic effects) were the most appropriate ones. In particular, they are characterized by the lowest global p-value (< 0.08), the highest R^2 (> 0.75), the highest R^2_{adj} ($= 0.59$), and the highest lack-of-fit p-value > 0.08 . This is considered a good result in view of the great variability in handling biological raw materials. The most significant coefficients (p-value < 0.05) of the factor of the second order model are those of the quadratic effect (p-value = 0.015) of the total enzymatic concentration and the linear effect (p-value = 0.019) of the

extraction time, followed by the linear effect (p-value = 0.041) of the total enzymatic concentration.

Optimization of the total enzymatic concentration and extraction time. The optimal values for the two factors for predicted maximum yield are identified by Response Surface Methodology. One can easily observe the quadratic effect of the total enzymatic concentration and the linear effect of the extraction time on the yield (see contoured response surface in Figure 3). According to the prediction profiling, the values for the total enzymatic concentration and the extraction time which maximized the yield are 2.85% and 10.9 hours, respectively (see red vertical line that indicates optimum value for each factor, in Figure 4). In these conditions, the predicted yield was $60.55 \pm 5.98\%$. Note that this value is similar to the maximum yield obtained experimentally (see essay 8, Table 2).

As the optimized extraction time lies at the upper extremity of the range of values, some more independent essays were performed in triplicate using extraction times of 8, 12 and 16 hours in order to statistically compare the experimental yields obtained. The results ($57.55 \pm 1.19\%$, $57.28 \pm 2.77\%$ and $56.03 \pm 1.43\%$, respectively) were analyzed by Analysis of Variance (ANOVA) with a significance level of 5% ($\alpha = 0.05$) and show that there is no statistical difference between the results (p-values > 0.05).

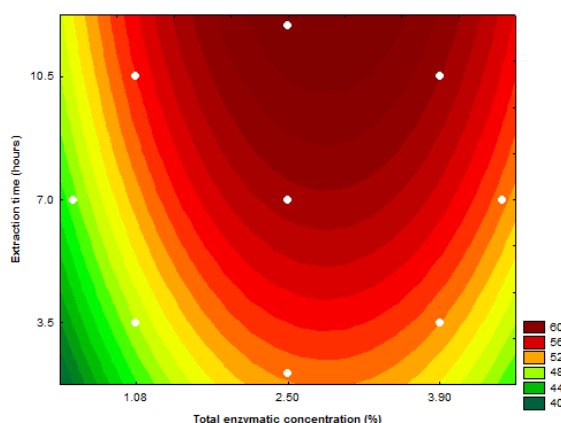


FIGURE 3. Contoured Response Surface of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the total enzymatic concentration (expressed in %) and the extraction time (expressed in hours) using the second order model for the two-factor Central Composite Design.

The essays were performed using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L Ultrazym AFP-L, respectively. The experimental design presents 11 experimental points in which the central point (2.5% and 7 hours) was performed in triplicate.

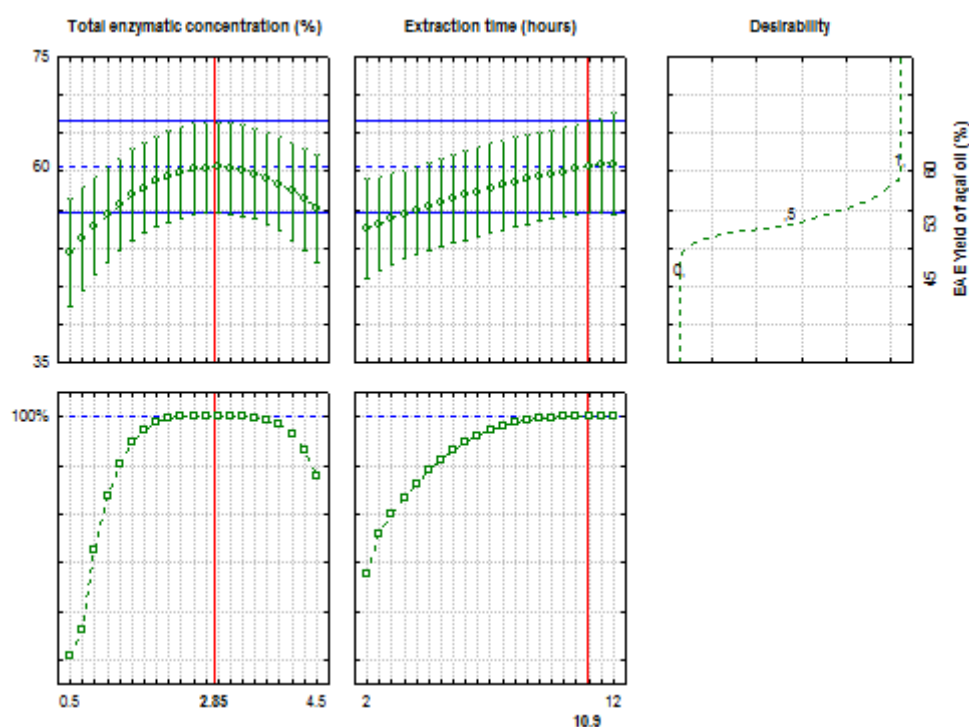


FIGURE 4. Prediction profiling and desirability function of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the total enzymatic concentration (expressed in %) and extraction time (expressed in hours) using the second order model for the two-factor Central Composite Design. The essays were performed using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L Ultrazym AFP-L, re

Understanding of the enzymatic phenomena.

As pointed out previously, the yield is significantly influenced by the quadratic effect of the total enzymatic concentration. The yield was enhanced when the total enzymatic concentration increased, because the enzymatic mixture of the three EP hydrolyzed the different polymers of the vegetable cell wall, which enable the release of the oil. However, once the total enzymatic concentration reached the maximum value, the yield slightly decreased with further increase in the total enzymatic concentration. This may be explained by possible enzyme aggregation caused by excess enzymes, which restricted their individual flexibility and activity, by the accumulation of intermediate products which inhibit the enzymatic activity, or by the stabilization of a fat emulsion in water by the enzymes which act as protein surfactants. Regarding the extraction time, the statistical treatment pointed out the significant linear effect of the extraction time, but the prediction profiling, the desirability function and the extra experimental essays showed that an increase in the extraction time up to 12 hours did not provide a significantly

($\alpha = 0.05$) higher yield compared to 8 hours. Some authors explained that a long extraction time might contribute to the depletion of the substrates and/or the product inhibition of enzymes (Zhang *et al.*, 2007; Li *et al.*, 2011), to a partial inactivation of the enzyme and/or to the adsorption of the oil onto the remaining solid fraction, or to the liberation of cellular components including phospholipids, glycolipids, and proteins, which form lipid-protein aggregates and emulsion stabilization (Nguyen *et al.*, 2020), consequently reducing the yield.

Comparison with the literature. Some papers have already used experimental designs for the identification of the optimal values of the total enzymatic concentration and the extraction time (among others) for the maximization of the yield of the oil extracted by the EAE process from different vegetable matrices, using cellulase, hemicellulase and/or pectinase (see Table 4).

Regarding the total enzymatic concentration, Xie *et al.* (2011), Mai *et al.* (2013), Nguyen *et al.* (2020) and Liu *et al.* (2020) also observed the significant quadratic effect of the total enzymatic concentra-

TABLE 4. Comparison of the ranges and optimal values for the total enzymatic concentration (%) and the extraction time (hours) suited by the Experimental Design for the maximization of the yield (%) of the oil extracted by the EAE process from different vegetable matrices, using cellulase, hemicellulase and/or pectinase.

Reference	Matrix	Enzymatic preparations			Methodology	Total enzymatic concentration (%)						Extraction time (hours)	L	Q	%
		Cellulase	Hemicell.	Pectinase		Range	Optimal	L	Q	Range	Optimal				
* Abdulkarim <i>et al.</i> 2006	Moringa seed	C	-	other	OFAT	0.5-2.5	>2			0-48	>36				74
* Zhang <i>et al.</i> 2007	Rapeseed	other	-	other	OFAT	0-5	>2.5			1-7	>4				90
* Tabatabei and Diosady, 2013	Yellow mustard flour	C	V	other	OFAT	-	3			-	3				76
Zhang <i>et al.</i> 2007	Rapeseed	other	-	other	RSM	0.5-1.5	1.5	X		1-3	3	X			75
Womeni <i>et al.</i> 2008	Bush mango kernel	-	V	other	RSM	0-2	>1.4	X		6.6-18.4	>14	X	X		83
Xie <i>et al.</i> 2011	Wheat germ	-	V	-	RSM	0.1-4	1.1		X	0.5-24	>5.25	X	X		66
* Mai <i>et al.</i> 2013	Gac fruit	other	-	other	RSM	5-25	14	X	X	1-3	2	X	X		80
Nguyen <i>et al.</i> 2020	Sacha Inchi Seed	other	-	-	RSM	2-6	4.5	X	X	2-6	5	X	X		95
Liu <i>et al.</i> 2020	Peanut	other	V	other	RSM	0.5-2	1.5	X	X	0.3-2.3	1.5		X		78

C=Cellulast 1.5L, U=Ulirazym AFP-L, V=Viscozyme L, OFAT=One Factor At a Time, RSM=Response Surface Methodology.

* References also present in Table 3. L. Linear factors. Q. Quadratic factors.

tion on yield. Other papers (Abdulkarim *et al.* 2006; Zhang *et al.* 2007 and Womeni *et al.*, 2008) showed a stabilization of the yield above a value of total enzymatic concentration (see Total enzymatic concentration (%), Table 4).

Regarding the enzymatic time, Zhang *et al.* 2007, Womeni *et al.* 2008, Xie *et al.* 2011, Mai *et al.* 2013 and Nguyen *et al.* 2020 also pointed out the significant linear effect of the extraction time, even if they showed that an increase in the extraction time up to the predicted optimal value did not provide a significant higher yield. This was also the conclusion of the papers of Abdulkarim *et al.* (2006); Zhang *et al.* (2007) and Liu *et al.* (2020) (see Extraction time (hours), Table 4).

4. CONCLUSIONS

In the present work, an efficient and sustainable enzymatic aqueous extraction process for açai oil is suggested. Its particularities are to perform the extraction from “in nature” wet açai decocts and using exclusively carbohydrases. Three enzymatic aqueous extraction parameters (i.e. proportion of three commercial carbohydrase enzymatic preparations, total enzymatic concentration and extraction time) were optimized using two successive Experimental Designs coupled to Response Surface Methodology. Under the optimum conditions identified (0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L and Ultrazym AFP-L, total enzymatic concentration: 2.85%, extraction time: 10.9 hours), the highest oil recovery yield of $60.55 \pm 5.98\%$ was estimated. This process allows satisfactory yields compared to conventional extraction methods, with the advantage of being carried out under environmentally-friendly conditions. Moreover, this technique is relatively accessible and easily implantable by Amazonian communities, and could help to create new market opportunities.

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REFERENCES

- Abdulkarim SM, Lai OM, Muhammad SKS, Long K, Ghazali HM. 2006. Use of enzymes to enhance oil recovery during aqueous extraction of moringa oleifera seed oil. *J. Food Lipids* **13**, 113–130. <https://doi.org/10.1111/j1745-4522200600038x>
- Batista CCR, Oliveira MS, Araújo ME, Rodrigues AMC, Botelho JRS, Filho APSS, Machado NT, Junior RNC. 2019. Supercritical CO₂ extraction of açai (Euterpe oleracea) berry oil: Yield fatty acids allelopathic activities and determination of phenolic and anthocyanins total compounds in the residual pulp. *J. Supercrit. Fluids* **107**, 364–369. <https://doi.org/10.1016/j.supflu.2015.10.006>
- Bichara CMG, Rogez H. 2011. Chapter 1—Açai (Euterpe oleracea Martius) In: Postharvest biology and technology of tropical and subtropical fruit: Açai to Citrus Cambridge: UK Press pp 1–26.
- Darnet S, Serra JL, Rodrigues AMC, Silva LHM. 2011. A high-performance liquid chromatography method to measure tocopherols in assai pulp (Euterpe oleracea). *Food Res. Int.* **44**, 2107–2111. <https://doi.org/10.1016/j.foodres.2010.12.039>
- Dela Cruz FB, Migo VP, Valência SA, Demafelis RB, Alfafara CG, Alcantara AJ. 2007. Enzyme mixture for the extraction of oil from seed of Vutalao (Calophyllum inophyllum). *Philipp J. Crop Sci.* **32** (3), 17–30.
- Domínguez H, Núñez MJ, Lema JM. 1994. Enzymatic pretreatment to enhance oil extraction from fruit and oilseeds: A review. *Food Chem.* **49**, 271–286. [https://doi.org/10.1016/0308-8146\(94\)90172-4](https://doi.org/10.1016/0308-8146(94)90172-4)
- Ferreira ES, Rogez HLG, Herman CANP. 2018 Effect of the combination of enzymatic preparations on the aqueous extraction yield of the oil from the pulp of Euterpe oleracea fruit. *Braz. J. Chem. Eng.* **35** (4), 1193–1201. <http://doi.org/10.1590/0104-663220180354s20170305>
- Li H, Song C, Zhou H, Wang N, Cao D. 2010. Optimization of the aqueous enzymatic extraction of wheat germ oil using Response Surface Methodology. *J. Am. Oil Chem. Soc.* **88** (6), 809–817. <https://doi.org/10.1007/s11746-010-1731-6>
- Liu C, Hao L, Chen F, Yang C. 2020. Study on extraction of peanut protein and oil bodies by aqueous enzymatic extraction and characterization of protein. *J. Chem.* **2020**, ID 5148967 11 p. <https://doi.org/10.1155/2020/5148967>

- Magalhães TSSA, Macedo PCO, Converti A, Lima AAD. 2020 The use of *Euterpe oleracea* Mart. as a new perspective for disease treatment and prevention. *Biomolecules* **10** (6), 813. <https://doi.org/10.3390/biom10060813>
- Lubrano C, Robin JR, Khaiat. 1994. A Fatty acids sterol and tocopherol composition of oil from the fruit mesocarp of six palm species in French Guiana. *OCL*.
- Mai HC, Truong V, Debaste F. 2013. Optimization of enzyme-aided extraction of oil rich in carotenoids from gac fruit (*Momordica cochinchinensis* Spreng). *Food Technol. Biotech.* **51** (4), 488–499.
- Nascimento RJS, Curi S, Assis RA, Freitas SP. 2008. Composição em ácidos graxos do óleo da polpa de açaí extraído com enzimas e com hexano. *Rev Bras Frutic* **30**(2):498–502.
- Nguyen HC, Vuong DP, Nguyen NTT, Nguyen NP, Su C-H, Wang F-M, Juan H-J. 2020. Aqueous enzymatic extraction of polyunsaturated fatty acid-rich sacha inchi (*Plukenetia volubilis* L.) seed oil: An eco-friendly approach. *LWT* **133**, 109992. <https://doi.org/10.1016/j.lwt.2020.109992>
- Pacheco-Palencia LA, Talcott SM, Talcott ST. 2008. Chemical composition antioxidant properties and thermal stability of a phytochemical enriched oil from açaí (*Euterpe oleracea* Mart). *J Agric. Food Chem.* **56**, 4631–4636. <https://doi.org/10.1021/jf800161u>
- Ricochon G, Muniglia L. 2010. Influence of enzymes on the oil extraction processes in aqueous media. *OCL* **17** (6), 356–359.
- Rosenthal A, Pyle DL, Niranjana K. 1996. Aqueous and enzymatic process for edible oil extraction. *Enzyme Microb. Technol.* **19**, 402–420. [https://doi.org/10.1016/S0141-0229\(96\)80004-F](https://doi.org/10.1016/S0141-0229(96)80004-F)
- Silva MP, Cunha VMB, Sousa SHB, Menezes EGO, Bezerra PN, Neto JTF, Filho GNR, Araújo ME, Carvalho Jr RN. 2019. Supercritical CO₂ extraction of lyophilized Açaí (*Euterpe oleracea* Mart.) pulp oil from three municipalities in the state of Pará, Brazil. *J. CO₂ Utilization* **31**, 226–234. <https://doi.org/10.1016/j.jcou.2019.03.019>
- Schauss AG. 2010. Açaí (*Euterpe oleracea* Mart): A macro and nutrient rich palm fruit from the Amazon rain forest with demonstrated bioactivities in vitro and in vivo. In D R Watson & V R Preedy (Eds) *Bioactive foods promotion health* (pp 479–490) Oxford: Academic Press.
- Silvamanya H, Jahim JM. 2015. Enhancement of palm oil extraction using cell wall degrading enzyme formulation. *Malaysian J. Anal. Sci.* **19** (1), 77–87.
- Skrovankova S, Sumczynski D, Mlcek J, Jurikova T, Sochor J. 2015 Bioactive compounds and antioxidant activity in different types of berries. *Int. J. Mol. Sci.* **16** (10), 24673–24706. <https://doi.org/10.3390/ijms161024673>
- Tabatabaei S, Diosady LL. 2013. Aqueous and enzymatic extraction processes for the production of food-grade proteins and industrial oil from dehulled yellow mustard flour. *Food Res. Int.* **52**, 547–556. <http://dx.doi.org/10.1016/j.foodres.2013.03.005>
- Womeni HM, Ndjouenkeu R, Kapseu C, Mbiapo FT, Parmentier M, Fanni J. 2008. Aqueous enzymatic oil extraction from *Irvingia gabonensis* seed kernels. *Eur. J. Lipid Sci. Technol.* **110**, 232–238. <https://doi.org/10.1002/ejlt.200700172>
- Xie M, Dunford NT, Goad C. 2011. Enzymatic extraction of wheat germ oil. *J. Am. Oil Chem. Soc.* **88**, 2015–2021. <https://doi.org/10.1007/s11746-011-1861-5>
- Yamaguchi KKL, Pereira LFR, Lamarão CV, Lima ES, Veiga-Junior VF. 2015. Amazon açaí: Chemistry and biological activities: A review. *Food Chem.* **179**, 137–151. <https://doi.org/10.1016/j.foodchem.201501055>
- Yusoff MM, Gordon MH, Niranjana K. 2015 Aqueous enzyme assisted oil extraction from oilseeds and emulsion de-emulsifying methods: A review. *Trends Food Sci. Technol.* **41**, 60–82. <https://doi.org/10.1016/j.tifs.2014.09.003>
- Zhang S, Wang Z, Xu SY. 2007. Optimization of the aqueous enzymatic extraction of rapeseed oil and protein hydrolysates. *J. Am. Oil Chem. Soc.* **84**, 97–105. <https://doi.org/10.1007/s11746-006-1004-6>