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OPTIMIZATION OF THE AQUEOUS ENZYMATIC EXTRACTION OF OIL FROM *OECOPETALUM MEXICANUM*

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— Abstract—

In the present work, the bromatological composition of the cacaté seed (*Oecopetalum mexicanum*) was analyzed and the process of aqueous enzymatic extraction of the oil was optimized. The enzymatic preparations Crystalzyme Cran and Cellulase were evaluated to determine their influence on the oil extraction yields of *O. mexicanum*. The experimental part was developed in two stages, the first one evaluated the effect of the variables type of enzyme, enzyme concentration, particle size and incubation time by means of an experimental L8 type Taguchi design, the second stage was to optimize those variables that influenced the oil yield, through a Box-Benhken design. The results of the bromatological analysis showed that the cacaté seed contains 39.25 ± 0.33% oil. An increase in oil extraction was observed using the Crystalzyme Cran enzymatic preparation at 50°C, with a solid:liquid ratio of 1:5. Optimal conditions for the extraction of *O. mexicanum* oil obtained by the Box-Benhken experimental design were agitation speed of 89 rpm, enzyme concentration of 0.5% and particle size 0.595 mm with a yield percentage of 150.

Keywords

Oecopetalum mexicanum; oil extraction; aqueous enzymatic process; comercial enzyme preparation.

Oilseeds are characterized by their content of high quality fats or oils, and the method used for their extraction is an important stage for their commercialization. There are several alternatives for the extraction of oil which have a direct effect on the extracted performance and the quality of oil obtained; being extraction by solvent and by pressing the most used processes at the industrial level (De Moura *et al.*, 2008; Latif and Anwar, 2008; Do and Sabatini, 2010; Li *et al.*, 2013). However, the use of solvents for oil extraction has been shown to have several drawbacks such as damage to the environment (Taha and Hassanein, 2007; Zhang *et al.*, 2010), low oil quality (Latif and Anwar, 2011), safety issues due to the use of solvents (Latif and Anwar, 2008) and low quality of the residual flour (Latif and Anwar, 2011; Li *et al.*, 2013) important reasons that have renewed interest in the search for eco-friendly alternative extraction processes (Li *et al.*, 2013; Mojtaba and Fardin, 2013).

In the last decade several researches have been described concerning the aqueous extraction of vegetable oils assisted by enzymes, such as the extraction of coconut oil (Mohammad *et al.*, 2015; Agarwal and Bosco, 2017), cotton (Taha and Hassanein, 2007), Moringa concanensis (Latif and Anwar, 2008), grapes (Guerra and Zúñiga, 2003), soybeans (Kapchie *et al.*, 2008; Kapchie *et al.*, 2010; Mohammad et al., 2015), olive (Ghodsvali *et al.*, 2009), palm oil (Teixeira *et al.*, 2013), among others.

Enzymatic aqueous extraction has emerged as a promising biotechnological tool, competent for the extraction of oil from various oleaginous materials (Latif and Anwar, 2011; Lianzhou *et al.*, 2011; Li *et al.*, 2013; Kumar *et al.*, 2017), offering several advantages compared to conventional extraction, due to the specificity of the enzymes and the moderate operating conditions, mainly their action at low temperatures (Soto *et al.*, 2008; Ahmadi *et al.*, 2013; Li *et al.*, 2013; Mojtaba and Fardin, 2013). The main function of the enzymes during the aqueous extraction of oil is to digest through hydrolysis the structure of the polysaccharides such as cellulose, hemicellulose and proto-pectin that form the cell wall of oilseeds increasing their permeability (Soto *et al.*, 2008; Kapchie *et al.*, 2010; Silvamany and Jahim, 2015) and, as a consequence, efficiency and extraction performance (Rathi *et al.*, 2012) of the oil and phenolic compounds retained in the cell wall matrix (Soto *et al.*, 2008) or proteins that form the cell membrane and lipid bodies (Taha and Hassanein, 2007; Latif and Anwar, 2008; Soto *et al.*, 2008) are improved.

At present there are commercial food grade enzymatic preparations with multiple activities such as cellulase, hemicellulase and pectinase; which are applied to oilseeds in order to hydrolyze the components of the tissues cell

wall (Ahmadi *et al*., 2013; Agarwal and Bosco 2014). The appropriate choice of enzyme preparation depends on the structural composition of the oilseed cell wall to be treated to ensure an efficient oil extraction (Yingyao *et al*., 2008; Amante *et al*., 2012; Rong *et al*., 2017). On the other hand, reaction conditions such as particle size, enzyme concentration and reaction time are factors that also influence the degree of hydrolysis and effectiveness of the process (Kumar *et al*., 2017).

The wide demand of vegetable oils for the purpose of use in the food and pharmaceutical industry has mainly, led to the search for new sources of native oilseeds as unconventional alternatives for the extraction and obtaining of oil. Mexico is a country that has a great wealth and biodiversity of native plants whose use potential is still unknown due to the few studies that have been carried out (SAGARPA, 2007). In this context, an arboreal plant known as cacaté (*Oecopetalum mexicanum*) is cultivated in Chiapas. It has been scarcely studied despite its attractive content of oil present in its seeds, and it could be considered as a viable unconventional alternative for oil production (Jimenez *et al*., 2013).

The *cacaté* or *cachichín* (*Oecopetalum mexicanum*) is an edible wild fruit that is distributed in the Mexican southeast (Veracruz, Chiapas and Tabasco) and Guatemala. A preliminary analysis of the cacaté seeds indicated that it is rich in oil, with contents similar to those presented by some oleaginous seeds recognized as soy, olive, avocado and corn (Ballinas *et al*., 2009).

Therefore, in the present work the process of aqueous enzymatic extraction was optimized to obtain oil from the cacaté seed.

MATERIALS AND METHODS

Raw material-Geographic location of the collection

The cacaté seeds (*Oecopetalum mexicanum*) were obtained in the local market of the municipality of Tecpatán, a town in the state of Chiapas, Mexico; whose geographical coordinates are 17°08'10'' N and 93°18'40 '' W, at an altitude of 320 meters above sea level. The climate of this municipality is warm-humid. To the north, it borders with the state of Tabasco and the municipality of Ostuacán, to the east with the municipalities of Francisco León, Copainalá and Ocotepec; to the south, with the municipalities of Berriozabal, Ocozocoautla and Cintalapa and to the west with the State of Veracruz (INEGI, 2005). A lot of 30 kg of cacaté seed was used, visually selected those that did not show apparent physical damage (Sant'Anna *et al*.,

2003). The seeds were kept in black polyethylene bags, stored in refrigeration at 4ºC until they were used (Ahmadi *et al*., 2013; Mohammad *et al*., 2015).

Grinding raw material

The cooled cacaté seeds were previously left at room temperature for 6 hours and then peeled manually, removing the testa and keeping the endosperm. The endosperms that corresponded to the fraction of interest, were ground in disk mills (Industrial Engineering) and later sieved in vibrating screen (Luheng Instrument Co.), obtaining a flour of varied granulometry. It was stored in amber bottles with a lid and kept refrigerated at 4ºC until used (Guerra and Zúñiga, 2003; Belén-Camacho *et al*., 2005).

Bromatological composition

The chemical characterization of the endosperm was evaluated by determining the bromatological composition moisture (AOAC 925.10), ash (AOAC 923.03), protein by the Kjeldahl method (AOAC 920.87), fat and oil by the Soxhlet method (AOAC 920.39), total fiber by acid and alkaline digestion (AOAC 985.29) and carbohydrates by weight difference (AOAC, 1990). All analyzes were performed in triplicate.

Aqueous-enzymatic extraction of oil

The aqueous-enzymatic extraction of the cacaté seed was done with 15g of the ground endosperm (Grasso *et al*., 2006) which was weighed in a 250 mL Erlenmeyer flask, the moisture content was adjusted to a solid:liquid ratio (w/v) 1:5 (Guerra and Zúñiga, 2003; Latif and Anwar, 2008). Subsequently, the enzyme preparation was added to an enzyme concentration: substrate according to Chart 1 (Guerra and Zúñiga, 2003), immediately after the sample was incubated for hydrolysis in a water bath with Felisa brand heating at 50ºC for the period established for each treatment (chart 1) as reported by Li *et al*., (2011). After the hydrolysis time, the sample contained in the flask was subjected to a boiling water bath for 5 min (Latif and Anwar, 2011), followed by a cold bath for 5 min to inactivate the enzyme.

The hydrolyzed sample was transferred to 50 mL Falcon tubes, centrifuged at 4,000 rpm for 20 min at 4°C (Latif and Anwar, 2008) in an Eppendorf brand centrifuge. The oil fraction (OilI) was recovered by decanting the centrifuged sample, storing at 8°C, in an amber flask. The aqueous phase was subjected to three washes with 15 mL of hexane, in order to recover the oil dispersed in the water, then the hexane was evaporated and the

fraction of oil (OilII) recovered was weighed and stored at 8°C in an amber bottle. All enzymatic hydrolysis treatments were carried out in triplicate, the control treatment was subjected to the same conditions except for the addition of enzyme, and the efficiency of the aqueous-enzymatic extraction process was compared with the extraction performance of the oil by solvent which was the control treatment, following the methodology described by the AOAC (1990).

Commercial enzyme preparations

The enzymatic preparations used in this study to improve the extraction efficiency of the cacaté oil were Crystalzyme Cran (CC) with cellulase, hemicellulase and pectinase activity of *Aspergillus niger* with optimum temperature of 40 to 60°C of the Valley Research laboratory and the preparation Cellulase 17600 (C17600) with cellulase activity of *Aspergillus niger*, optimum temperature of 40 to 60°C, both are considered GRAS enzymatic preparations (Generally Recognized As Safe) by the FDA (Food and Drug Administration) (Spök, 2006).

L8 Taguchi Experimental design

The effects of the variables type of enzyme, enzyme concentration, time, and particle size; were evaluated using an L8 Taguchi statistical design. The experimental work developed considers four factors: one qualitative (type of enzyme) and three quantitative factors (enzyme concentration, time and particle size) evaluating each independent variable in two levels (chart 1). The extraction yield, calculated by the following formula, was evaluated as a response variable.

$$\text{Performance } (\%) = \frac{\text{Oil}_{I} + \text{Oil}_{II}}{\text{Oil}_{T}} \times 100 \quad (1)$$

Where:

Oil_I is the oil phase recovered after centrifugation,
Oil_{II} is the oil recovered in the aqueous phase after washing with hexane,
Oil_T is the oil extracted with commercial hexane by extraction with Soxhlet.

Statistically significant treatments were evaluated using the analysis of variance (simple ANOVA) with a confidence level of 95% (Montgomery, 2001). The treatments that showed significant difference were analyzed through the Tukey test at 5% for the comparison of means (Latif and Anwar, 2008). The

data obtained were analyzed using the statistical software STATGRAPHICS Plus version 5.1. The treatment that generated optimal yields was used to establish the experimental design in the optimization.

Chart 1. Taguchi experimental design

Treatment	Commercial enzymatic preparation	Concentration of the enzyme preparation (%)	Particle size (mm)	Time (h)
1	CC	0.5	1.19	4
2	CC	0.5	2.38	8
3	CC	1.0	1.19	8
4	CC	1.0	2.38	4
5	CC-C17600	0.5	1.19	8
6	CC-C17600	0.5	2.38	4
7	CC-C17600	1.0	1.19	4
8	CC-C17600	1.0	2.38	8

Optimization of the operation variables

The variables that influenced the extraction performance of oil in the L8 Taguchi experimental design were subsequently optimized by the Response Surface Methodology (MSR), using a Box-Benhken experimental design (chart 2). The variables considered were agitation speed, enzyme concentration and particle size, evaluating each variable in three levels. The natural and coded levels of the independent variables used in the experimental design are shown in Chart 3.

Chart 2. Box-Benhken experimental design

Treatment	Agitation speed (rpm)	Enzyme concentration (%)	Particle size (mm)
1	0	0	0
2	-1	-1	0
3	1	-1	0
4	-1	1	0
5	1	1	0
6	-1	0	-1
7	1	0	-1
8	0	0	0
9	-1	0	1
10	1	0	1
11	0	-1	-1

12	0	1	-1
13	0	-1	1
14	0	1	1
15	0	0	0

Chart 3. Levels of the Box-Behnken design independent variables

Independent variable	Levels		
	-1	**0**	**1**
Particlle size [a] (mm)	0.595	0.8	1.19
Agitation speed [b] (rpm)	60	80	100
Enzyme concentration [c] (% v/v)	0.5	1.0	1.5

a = Guerra y Zúñiga (2003), b = Grasso *et al*. (2006), c = Latif y Anwar (2008)

The methodology of the response surface allowed determining the optimum combination of particle size, agitation speed, enzyme concentration that maximizes the extraction performance of the oil. The total treatment was 15 with three repetitions. The statistically significant effects were evaluated using the analysis of variance (ANOVA) at a level of 95% confidence ($P < 0.05$) (Montgomery, 2001; Latif and Anwar, 2008). The data obtained were analyzed using the statistical software STATGRAPHICS Plus version 5.1.

RESULTS AND DISCUSSION

General characteristics

The yields of the fractions that make up the *Oecopetalum mexicanum* seed are shown in chart 4. It should be noted that the major fraction corresponds to the endosperm that represented 54.91% of the total weight of the seed, and it is the fraction of interest as oleaginous matter.

Chart 4. Cacaté seeds fractions performance

Fraction	Proportion (%, w/w)
Full seed	100
Endosperm	54.91
Exocarp	45.09

Bromatological composition of the cacaté seed

The endosperm of the cacaté seed is characterized by a moisture content of 64.79 ± 0.20%, this value is relatively high compared to that reported by Cruz (2004) of seeds collected in the municipality of Ocozocoautla (47.26%) and by Ballinas *et al.* (2009) (52.6%) for seeds collected in the municipality of Tapilula. The difference in moisture content may be due to the geographic location of the crops, the harvest season, weather conditions, among other factors (Belén *et al*., 2001).

It is observed in chart 5 that the cacaté seed used in this study stood out for its attractive content in fat, protein and fiber, with 39.25, 12.59 and 4.25% respectively; these results are consistent with those obtained by Ballinas *et al.* (2009) for cacaté seeds collected in the municipality of Tapilula, Chiapas reporting a fat content of 35%, protein 13.24% and fiber of 4.15%. Centurion *et al.* (2000) reported fat content of 30.7% and protein of 8.0% of a cacaté seed harvested in the municipality of Tlacotlalpa, Tabasco. The variation observed in the fat content can be attributed to the extraction conditions of the oil (Solís-Fuentes *et al*., 2001), in addition the composition of the seed is influenced by the maturity of the fruit (Belén *et al*., 2001), the geographic location of the collection area, the harvest season and climatic changes (Belén *et al*., 2001; Matos and Acuña, 2010).

Chart 5. Bromatological composition of the cacaté seed

Compound	Content (g/100 g)
Ash	2.30±0.13
Fat	39.25±0.33
Protein	12.59±0.10
Raw fiber	4.25±0.16
Carbs	41.61±0.16

Fuente: Data reported on dry basis

Note: The results presented correspond to the mean ± SD (n=3)

The protein content of the cacaté is comparable with that of the amaranth (15%) and higher than the average reported for wheat (10.6%), corn (11%) and rice (7.4%) (Belén *et al*., 2001) The fiber content found for the cacaté seed (4.25%) is similar to the one reported for the sunflower (3.7%) (Badr and Sitohy, 1992) and *Moringa oleifera* (4.2%) (Compaoré *et al*., 2011), but lower to the reported for sesame (11.2%) and canola (7%) (Belén-Camacho *et al*., 2005), although the applied method does not reveal the nature of the

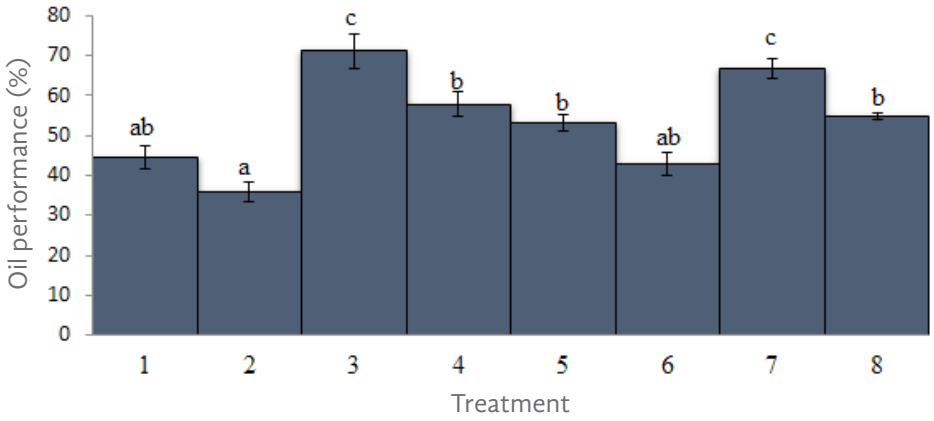
fibers of the cacaté seed, the presence of dietary fiber is important given the relationship with the prevention and control of cardiovascular diseases, diabetes and intestinal cancer (Belén *et al.*, 2001). On the other hand, the fat content was higher than the average of cereals (5%) (Belén *et al*., 2001) When compared to conventional oilseeds, it exceeds sesame (30%), cotton (22%) and soybeans (18%) (Belén-Camacho *et al*., 2005) and is lower than the one reported for canola (43%), *Moringa oleifera* (43.5%) (Compaoré *et al*., 2011), and sunflower (45%) (Badr and Sitohy, 1992), because of its attractive oil content, the cacaté seed could be considered an important unconventional source for harvesting of oil.

The protein and fiber content found in the cacaté seed makes it possible to derive that the byproduct resulting from the extraction of the oil could be used in the formulation of foods destined for human consumption or animal consumption, considering the beneficial effects for health that lead to the consume these compounds.

Enzymatic aqueous extraction of the oil using the Taguchi experimental design (L_8)

Image 1 shows the oil yields obtained in each treatment of the Taguchi experimental design. The yield of extracted oil varied from 35.9 to 71.1% according to the conditions of the enzymatic treatment, these values were higher than that obtained with the control treatment using hexane, which was 34.25 ± 2.00%. The maximum yield of extracted oil was obtained in the treatments T3 and T7, made with the enzymatic preparation Crystalzyme Cran (71.06 ± 4.37%) and the combination of Crystalzyme Cran-Cellulase 17600 (ratio 1:1) (66.68 ± 2.49%) respectively; statistically there was no significant difference ($p < 0.05$) between these two treatments. Based on these results, the enzymatic preparation Crystalzyme Cran was selected, with enzymatic activity cellulase, hemicellulase and pectinase which, due to its hydrolytic action, acts by breaking or destabilizing the structure of the cell wall of the cotyledon, promoting the solubilization of different components, mainly polysaccharides (Soto *et al*., 2008) making it more permeable (Silvamany and Jahim, 2015), which improves oil extraction yields. The saccharification of the cell wall polysaccharides is carried out with a mixture of cellulase enzymes and pectinases, also containing hemicellulolytic activity, the enzymes increase the surface area of contact with the solid particles increasing the extraction of the oleosome (Kapchie *et al*., 2010)

Image 1. Cacaté oil extraction performance, according to the Taguchi experimental design



The variables particle size (Image 2) and enzyme concentration (Image 3) presented a statistically significant difference ($p < 0.05$) on the response variable. An increase in particle size from 1.19 mm to 2.38 mm a significant decrease in the yield of extracted oil is observed, very close values of particle size of 1.5 mm in diameter was reported as adequate but not optimal for oil extraction (Matos and Acuña 2010). When increasing the concentration of enzyme (1% v / v), the yield of extracted oil increased reaching up to 65% (Image 3), this is attributed to the fact that at higher concentrations of enzymes, more active sites are available to interact with substrates, resulting in an increase in oil yield (Li *et al*., 2011).

Image 2. Means and 95.0 percentages LSD intervals for particle size

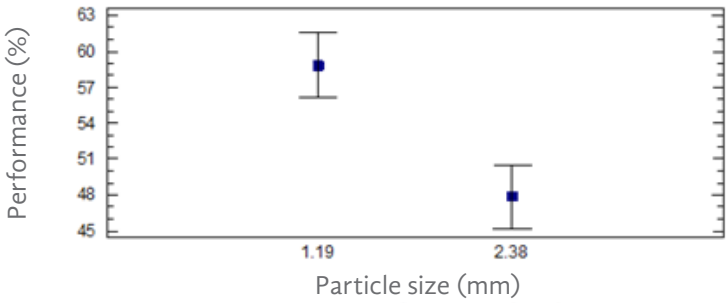
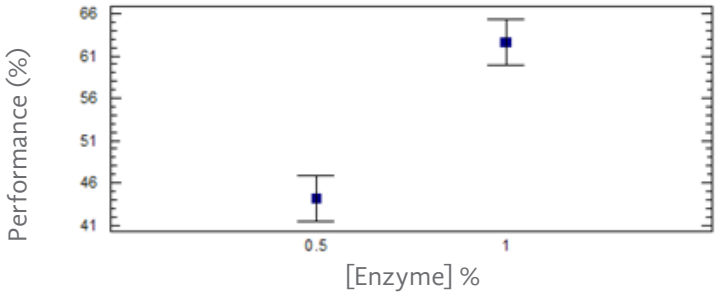


Image 3. Means and 95.0 percentages LSD intervals for enzyme concentration



The Taguchi experimental design allowed evaluating the effect of the enzyme concentration, particle size, incubation time and type of enzyme on the extracted oil yield. In Image 4 it is observed by the Pareto graph that the factors concentration of enzyme (P=0.0127) and particle size (P=0.0451) showed significant effect on oil extraction performance. The enzyme concentration induces a positive effect, while the particle size induces a negative effect (Image 4). The above is confirmed by the results of analysis of variance presented in chart 6.

Image 4. Standardized Pareto graphic for performance (means)

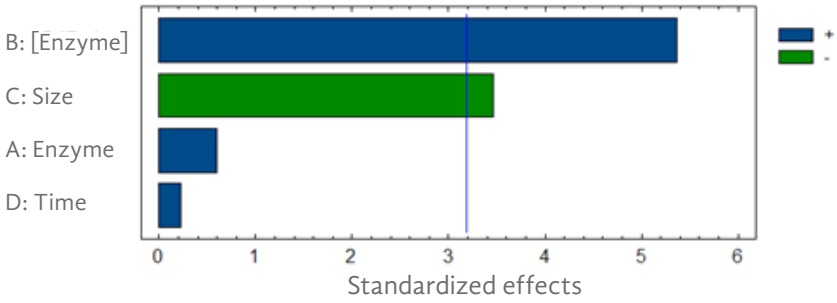


Chart 6. Variance analysis for the performance of cacaté oil

Source	Sum of Squares	Gl	Mean Square	F-Ratio	P-Value
A:Enzyme	1.35963	1	1.35963	0.36	0.5920
B:[Enzyme]	109.516	1	109.516	28.81	0.0127
C:Size	38.4708	1	38.4708	10.12	0.0451
D:Time	0.18635	1	0.18635	0.05	0.8390
Total error	11.405	3	3.80166		
Total (corr.)	160.938	7			

Optimization of the operation variables

The design of Box-Behnken to find the optimal conditions of enzyme concentration, particle size and stirring speed that maximizes oil extraction performance is presented in Chart 2. The Pareto graphic (Image 5) shows that only the Stirring speed had a significant effect on the oil extraction performance, which is verified with the results of the analysis of variance (chart 7) that showed significant statistical difference ($p < 0.05$) with a value of $P=0.0037$. The particle size, enzyme concentration and all interactions had no significant effect on the yield of extracted oil (Image 5 and Chart 7).

Image 5. Pareto graph for oil extraction performance

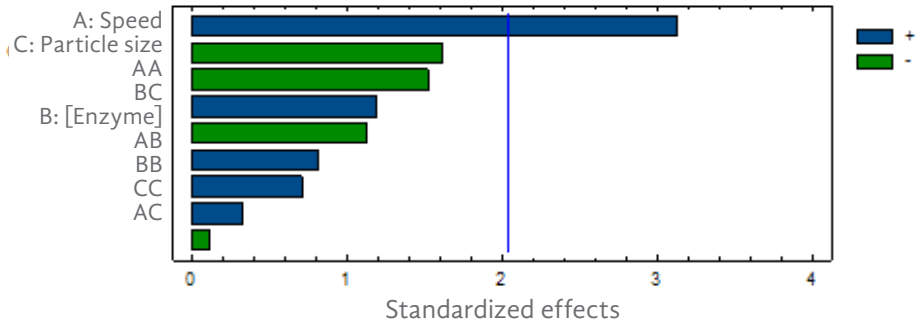


Chart 7. Variance analysis for oil performance

Source	Sum of Squares	Gl	Mean Square	F-Ratio	P-Value
A:Speed	8122.96	1	8122.96	9.76	0.0037
B:[Enzyme]	1047.67	1	1047.67	1.26	0.2700
C:Particle size	2180.49	1	2180.49	2.62	0.1150
AA	1931.26	1	1931.26	2.32	0.1372
AB	543.706	1	543.706	0.65	0.4247
AC	10.0631	1	10.0631	0.01	0.9131
BB	425.489	1	425.489	0.51	0.4796
BC	1190.0	1	1190.0	1.43	0.2403
CC	88.2608	1	88.2608	0.11	0.7467
Blocks	54.3765	2	27.1882	0.33	0.9679
Total error	27462.4	33	832.193		
Total (corr.)	43246.4	44			

According to the mathematical model, the maximum predicted yield of extracted oil was 150.82% with an R2 of 89.73%, under the optimum conditions of

agitation speed 80 rpm, enzyme concentration 0.5% and particle size 0.595 mm. The validation of the model was performed by repeating the experiment in triplicate under optimal conditions, obtaining an experimental oil extraction yield of 151.54%, this is a prediction error of 0.0048%; which indicates a satisfactory adjustment of the equation with the experimental data and suggests that the optimized model was adequate and effective.

The percentage of the oil performance was adjusted with the model:

$$Performance\ (\%) = 22.4836 + 5.66577A - 176.395B - 143.603C - 0.0330104A^2 + 0.673118A \cdot B - 0.153907A \cdot C + 24.791B^2 + 66.9462B \cdot C + 31.8934C^2 \quad (2)$$

Where, A is the agitation speed (rpm), B is the enzyme concentration (%) and C is the particle size (mm).

The equation of the Box-Behnken model (Ec.2) is represented graphically in Images 6 and 7. Considering the constant concentration of enzyme (Image 6), the response surface graph shows that at low values of agitation the yields of extraction are low, this because adequate diffusion of the enzyme on its substrate is not allowed; however, as the stirring speed increases, the oil yield is higher until reaching a speed in which there is a decrease in the performance which is attributed to the fact that the greater the agitation, the emulsion formation is allowed or favored preventing the enzyme-substrate contact (Sharma *et al*., 2002). It is observed (Image 6) a maximum oil extraction performance of 127 to 133% using agitation speed in the middle range and smaller particle size. These results agree with what was reported by (Ahmadi *et al*., 2013) who observed an increase in oil recovery by increasing the mixing speed from 40 to 80 rpm while the recovery was reduced by increasing the agitation by more than 80 rpm. The performance achieved can be attributed.

Considering the stirring speed (Image 7) it is observed in the response surface graph that at the lowest enzyme concentration and the smallest particle size, the oil extraction yield was maximized up to 133 to 139%.

Image 6. Response surface estimated for [Enzyme] = 1.0%

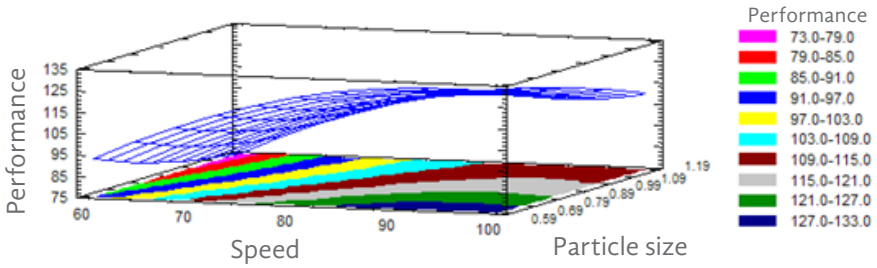
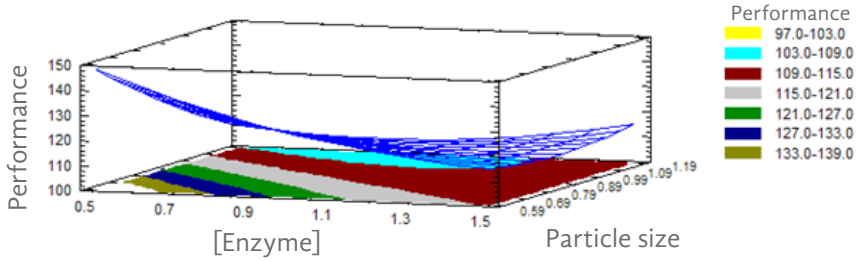


Image 7. Response surface estimated for speed = 80 rpm



CONCLUSION

The cacaté seed is a recommended raw material for the extraction of oil, as a product of interest, in addition for its protein content the flour could be used in the formulation of food intended for human or animal.

The enzymatic treatment of the cacaté fruits endosperm (*Oecopetalum mexicanum*) was an efficient method to improve the extraction yields of the oil.

The process of standardization of the operating conditions allowed establishing the parameters that influence the extraction performance, being these temperature, particle size, solid: liquid ratio and type of enzyme. On the other hand, the Box-Benhken experimental design showed optimal conditions: agitation speed=89 rpm, enzyme concentration = 0.5% and particle size 0.595 mm with an optimum oil performance of 150.

The model has an error of prediction of oil extraction performance of 0.0048%, indicating that the model predicts in a 99.52% the response variable.

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