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Optimization of pectinolytic hydrolysis in Caatinga passion fruit wine must with commercial pectinase, according to the central composite rotatable design approach

Otimização da hidrolise pectinolítica em mosto de maracujá da Caatinga com pectinase comercial, de acordo com a abordagem do Delineamento Composto Central Rotacional

Otimización de la hidrólisis pectinolítica en mosto de vino de maracuyá Caatinga com pectinasa comercial, según el enfoque de diseño rotatório compuesto central

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Abstract
The *Passiflora cincinnata* Mast. has high flavor and potential for wine and other alcoholic beverages production. However, for wine production, the presence of pectin becomes undesirable, as it influences the efficiency of the fermentation process and the clarity of the final product. The objective of this study was to optimize the pectin hydrolysis process in Caatinga passion fruit must for wine production with pectinolytic enzymes, using a Central Composite Rotatable Design (CCRD). The 2^3 factorial CCRD, E01 to E17 assays, was applied with six axial tests and three replications in the central point. Caatinga passion fruit must was obtained with pulp and distilled water (40:60 proporcion). Commercial enzyme with high concentration of pectinase was tested. Concentration of enzyme (0.014-0.056 g L^{-1}), working temperature (43-57°C) and reaction time (12-139min) were the independent variables in the optimization process; and the soluble pectin content (mg $100g^{-1}$) corresponded to a process-dependent variable. Prior to the beginning of the assays, in order to provide the optimum working pH range for the pectinase, correction of the must pH to 3.9. The initial Caatinga passion fruit wine must had 24.06 mg $100g^{-1}$ of soluble pectin in its composition. After the optimization using CCRD, the treatments presented pectin contents ranging from 6.81 (E16: 0.014g L^{-1}/50°C/75min) to 0.00 mg 100 g^{-1} (E05: 0.05 g L^{-1}/45°C/ 30min and E06: 0.05 g L^{-1}/45°C/120min). According to the results, to a satisfactory process of pectin hydrolysis in Caatinga passion fruit wine must be added 0.05 g L^{-1} of pectinase at 45°C for 30min.
Keywords: Caatinga biome; DOE; Factorial design; *Passiflora cincinnata* Mast.; Pectin.

Resumo
O *Passiflora cincinnata* Mast. apresenta elevado sabor e potencial para produção de vinho e outras bebidas alcoólicas. No entanto, para a produção de vinho, a presença de pectina torna-se indesejável, pois influencia na eficiência do processo de fermentação e na limpidez do produto final. O objetivo deste estudo foi otimizar o processo de hidrólise da pectina em mosto de maracujá da Caatinga para produção de vinho com enzimas pectinolíticas, utilizando um Delineamento Composto Central Rotacional (DCCR). O DCCR fatorial 2^3, ensaios E01 a E17, foi aplicado com seis

testes axiais e três repetições no ponto central. O mosto de maracujá da Caatinga foi obtido com polpa e água destilada (proporção 40:60). Enzima comercial com alta concentração de pectinase foi testada. Concentração da enzima (0,014-0,056 g L^{-1}), temperatura de trabalho (43-57°C) e tempo de reação (12-139min) foram as variáveis independentes no processo de otimização; e o teor de pectina solúvel (mg $100g^{-1}$) correspondeu a uma variável dependente do processo. Antes do início dos ensaios, a fim de fornecer a faixa de pH de trabalho ideal para a pectinase, corrigiu-se o pH do mosto para 3,9. O mosto inicial de maracujá da Caatinga continha 24,06 mg $100g^{-1}$ de pectina solúvel em sua composição. Após a otimização com DCCR, os tratamentos apresentaram teores de pectina variando de 6,81 (E16: 0,014g L^{-1}/50°C/75min) a 0,00 mg 100 g^{-1} (E05: 0,05 g L-1/45°C/30min) e E06: 0,05 g L^{-1}/45°C/120min). De acordo com os resultados, para um processo satisfatório de hidrólise de pectina em vinho de maracujá da Caatinga deve-se adicionar 0,05 g L^{-1} de pectinase a 45°C por 30min.
Palavras-chave: Bioma Caatinga; DOE; Design fatorial; *Passiflora cincinnata* Mast.; Pectina.

Resumen
El *Passiflora cincinnata* Mast. tiene alto sabor y potencial para la producción de vino y otras bebidas alcohólicas. Sin embargo, para la producción de vino, la presencia de pectina se vuelve indeseable, ya que influye en la eficiencia del proceso de fermentación y la claridad del producto final. El objetivo de este estudio fue optimizar el proceso de hidrólisis de pectina en mosto de maracuyá Caatinga para la producción de vino con enzimas pectinolíticas, utilizando un Diseño Rotativo Compuesto Central (DRCC). Se aplicó el ensayo factorial 2^3 DRCC, E01 a E17, con seis pruebas axiales y tres repeticiones en el punto central. El mosto de maracuyá caatinga se obtuvo con pulpa y agua destilada (proporción 40:60). Se probó enzima comercial con alta concentración de pectinasa. La concentración de enzima (0.014-0.056 g L^{-1}), la temperatura de trabajo (43-57°C) y el tiempo de reacción (12-139min) fueron las variables independientes en el proceso de optimización; y el contenido de pectina soluble (mg $100g^{-1}$) correspondió a una variable dependiente del proceso. Previo al inicio de los ensayos, con el fin de proporcionar el rango de pH óptimo de trabajo para la pectinasa, corrección del pH del mosto a 3,9. El mosto de maracuyá Caatinga inicial tenía en su composición 24,06 mg $100g^{-1}$ de pectina soluble. Luego de la optimización mediante DRCC, los tratamientos presentaron contenidos de pectina que van desde 6.81 (E16: 0.014g L^{-1}/50°C/75min) hasta 0.00 mg 100 g^{-1} (E05: 0.05 g L^{-1}/45°C/30min y E06: 0,05 g L^{-1}/45°C/120min). De acuerdo a los resultados, a un proceso satisfactorio de hidrólisis de pectina en vino de maracuyá Caatinga se le debe adicionar 0.05 g L^{-1} de pectinasa a 45°C por 30min.
Palabras clave: Bioma Caatinga; DOE; Diseño factorial; *Passiflora cincinnata* Mast.; Pectina.

1. Introduction

Passion fruit is a tropical plant with wide genetic variability, belonging to the *Passifloraceae* family. It is formed of 20 genera and 630 species, of which the genus *Passiflora* is economically the most important, composed of 24 sub-genera and from 400 to 530 species, of which 150 to 200 are native to Brazil (Cervi, 2006; Amorim et al., 2013; Carvalho et al., 2017).

The native Brazilian fruit, or Caatinga passion fruit (*Passiflora cincinnata* Mast.), which occurs frequently and spontaneously in regions with Caatinga Bioma, is a climacteric fruit presenting a lengthy ripening period after harvest, and when ripe, has a greenish-yellow colored skin, soluble solids content from 8 to 13%, pH value varying from 2.0 to 3.0, approximately 3.0% of acidity and an elevated pectin content (Aidar et al., 2016; Jesus & Faleiro, 2016; Araújo et al., 2016; Araújo et al., 2018; Santos et al., 2020b). The fruit also has high antioxidant activity, vitamin C and other bioactive compounds contents, making it very attractive to consumers (Silva et al., 2020; Santos et al., 2021a; Santos et al., 2021b). The pectin present in passion fruit is constituted of 76 to 78% galacturonic acid, 9% methoxyl groups and a small amount of galactose and arabinose (Manica, 1981).

It is of great economic importance for farmers in the agro-industries in the Caatinga biome of Brazil and shows an elevated potential for the nutritional improvement of alcoholic beverages, as Caating Passion fruit wine (Santos et al., 2021a). However, when beverages are elaborated from fruit juices, the presence of insoluble matter such as pectin is undesirable during processing, since this substance forms a colloid which retards or avoids sedimentation of the solid particles which confer, above all, turbidity to the beverage, thus influencing the efficiency of the clarification process (Uenojo & Pastore, 2007; Moreno-Arribas & Polo, 2009).

Pectins are structural polysaccharides present in vegetable cells, found in different amounts and with different compositions, constituted mainly of galacturonic acid and neutral sugars such as rhamnose, galactose, arabinose and xylose (May, 2000; Yapo et al., 2006). This substance is easily precipitated in the presence of organic solvents or co-solvents,

alcohols such as ethanol or methanol usually being employed, due to the insolubility of the pectic substances in these solvents (Liu et al., 2006).

In alcoholic beverages, apart from the influences cited above for fruit juices, pectin also influences the fermentative process, since its presence can promote the liberation of methanol (methyl alcohol) from the methylated polygalacturonic acids during processing of the beverage (Canteri et al., 2012; Reis et al., 2020). Methanol is a secondary compound considered undesirable in alcoholic beverages and can cause undesirable changes in the flavor of the beverage as well as harming consumer health, due to its toxic properties (Reis et al., 2020; Gomes et al., 2020). Its production is undesirable in wines, and if it occurs, should not exceed the limit of 300 mg L^{-1} in white or sparkling wines (Brasil, 2018). Thus, pectinolytic enzymes are widely used in food industries in order to reduce these effects.

During enzymatic treatment, the pectinolytic enzymes have the function of depolymerizing or de-esterifying the pectic compounds, hydrolyzing the glycosidic bonds along the carbon chain of the pectin, aiding the formation of pectin-protein flakes and hence reducing their water-holding capacity, consequently reducing the viscosity of the juice due to the water liberated in the system (Uenojo & Pastore, 2007; Ninga et al., 2021).

The Experimental Design (ED) can be carried out in a planned way, whereby the factors or independent variables are fitted to evaluate their impact on the variable response (dependent), and in the case of two or three independent variables, a Central Composite Rotatable Design (CCRD) is recommended (Rodrigues & Iemma, 2014).

Given the above, the objective of this study was to optimize the pectin hydrolysis process in Caatinga passion fruit must for wine production with pectinolytic enzymes addtion, using a Central Composite Rotatable Design.

2. Methodology

2.1 Raw material

Caatinga passion fruits in an intermediate ripeness stage of maturation were manually harvested from the Embrapa Experimental Field (Petrolina, Brazil). The fruits were visually selected considering a firm skin, no squashed areas or skin cuts and the apparent absence of microbial contamination. The fruits were stored for sixty days after harvest, at a temperature of 28 ± 2ºC, until the skin reached a greenish-yellow color. In sequence, the fruits were sanitized, first washing under running water and then immersing in a 200 mg L^{-1} sodium hypochlorite solution for 15 min, before rinsing under running water. The pulp and seeds were then removed from the skin and pulped in an electric pulping machine with a size 10 mesh sieve (Macanuda, model DMJI-05, Brazil).

The fruit pulp was characterized in triplicate analysis, according to the methodologies proposed by AOAC (2012) for: soluble solids - direct reading using a portable refractometer (Atago, Pocket Refractometer model PAL-3), pH - direct reading of the samples using a pH meter (Hanna Instruments, model HI 2221) and titratable acidity - titration with 0.1M NaOH. Additionally, was determined in the fruit pulp, reducing sugars - by the UV spectrometer (540 nm) using the 3,5-dinitro-salicílico (DNS) acid method (Miller, 1959), and soluble pectin - by the UV spectrometer (520 nm) using the m-hydroxydiphenyl method (McReady & MacComb, 1952).

2.2 Enzyme

The enzyme tested was a commercial enzyme - Endozym Pectofruit (AEB, Paraná - Brazil), commercially characterized as a purified enzyme, extracted from *Aspergillus*, preparation with a high concentration of pectinase, produced to be used in the depectinization of concentrated fruit juices.

2.3 Obtaining the Caatinga passion fruit wine must

The Caatinga passion fruit wine must was obtained with 40% of pulp and 60% of distilled water. Prior to the beginning of the assays tested, in order to provide the optimum working pH range for the pectinase, correction of the must pH to 3.9 was performed using a deacidifying complex DEACID® (AEB, Paraná - Brazil), composed by ammonium salts. Besides improving pectinase activity, the dilution and pH correction also aimed to correct the must acidity in order to reach the values stablished by the fruit beverage legislation that is stablished to be between 50 mEq L^{-1} to 130 mEq L^{-1} (Brasil, 2012).

2.4 Experimental design

A 2^3 factorial starshaped CCRD was used with six axial trials and three repetitions at the central point, giving a total of seventeen trials (E1 to E17). The independent variables were the working temperature (ºC), action time (min) and pectinase concentration (g hL^{-1}) and the dependent variable was the soluble pectin content (mg $100g^{-1}$). Tables 1 and 2 show the levels and values of the independent variables used and the experimental design matrix, respectively. The levels studied for these variables were determined based on the conditions cited on the datasheet of the commercial enzyme used (Endozym Pectofruit).

A total of 250 mL of passion fruit must was used for each trial and carried out with the aid of a digital pH meter (Hanna Instruments, model HI 2221), digital analytical balance (CRYSTAL 200 model CAL, Gilbertinni), water bath with openings and digital temperature control (model Q334M-28, Quimis) and a digital thermometer (AK05, Akso). After the predefined hydrolysis times for the trials, the must was cooled in an ice slurry bath to a temperature of 30ºC and then left to rest under refrigeration (7±1°C) for 24h before analyzing the soluble pectin content.

Table 1. Levels and values of the independent variables used in the Central Composite Rotatable Design - CCRD.

		Independente Variable		
	levels	**Time (min.)**	**Temperature (°C)**	**Pectinolytic enzyme concentration (g L^{-1})**
Factorial Planning	-1	30	45	0.02
	+1	120	55	0.05
Axial Points	- α	12	43	0.014
(star configuration)	+ α	139	57	0.056
Central Point	0	75	50	0.035

Source: Authors.

Table 2. Codified matrix of the complete 2^3 factorial experimental design plus axial points applied to Caatinga passion fruit wine must.

	Coded Matrix		
Run	**Time (min.)**	**Temperature (°C)**	**Pectinolytic enzyme concentration (g L^{-1})**
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9 (axial)	-1.68	0	0
10 (axial)	+1.68	0	0
11 (axial)	0	-1.68	0
12 (axial)	0	+1.68	0
13 (axial)	0	0	-1.68
14 (axial)	0	0	+1.68
15 (C)	0	0	0
16 (C)	0	0	0
17 (C)	0	0	0

Source: Rodrigues & Iemma (2014), adapted by the Authors.

2.5 Determination of pectin

Soluble pectin (mg $100g^{-1}$) was determined by reading the sample extract (extracted with 95% and 75% ethanol) with a solution 0.0125 M of tetraborate/sulfuric acid and 0.15% m-hydroxyphenyl in a refractometer at 520 nm according to the technique used by McReady & MacComb (1952). The response of the DOE (Design of Experiments) expressed the percent reduction of pectin in the must, considering the initial pectin content before hydrolysis as 100%.

The quantitative pectin contens in the samples were determined in the *in natura* Caatinga passion fruit pulp and in the wine must before and after enzymatic hydrolysis. The Response Pectin Reducion (RPR) was obtained through of the equation 1, that is:

$$RPR\,(\%) = ((PI - PF)/PI\,)*100 \qquad (1)$$

where:

PI - pectin content in the must before hydrolysis;

PF - pectin content in the hydrolyzed must.

2.6 Statistical Analysis

The results were statistically investigated using the software STATISTICA version 10.0 (StatSoft Inc®, USA), with 95% significance level, for analysis of effects and verification of the empirical model through the regression coefficient and ANOVA.

3. Results and Discussion

The parameters evaluated in the physicochemical composition of the Caatinga passion fruit pulp indicated a soluble solids (SS) content of 9.8%, pH in the range of 2.80, titratable acidity (TA) of 4.49% (expressed in citric acid), 32.13 g L^{-1} of reducing sugars (RS) and pectin content of 63.42 mg 100 g^{-1}, as shown in Table 3.

Table 3. Physicochemical composition of Caatinga passion fruit pulp.

Physico-chemical parameters	Caatinga passion fruit pulp[1]
Soluble solids (%)	9.8±0.00
pH	2.80±0.00
Titratable acidity (% citric acid)	4.49±0.02
Ratio (SS/TA)	2.18±0.01
Reducing sugar (g L^{-1})	32.13±0.04
Pectin (mg 100g^{-1})	63.42±3.32

(1) Values are expressed as mean ± standard deviation (*n*=3). Source: Authors.

The physicochemical composition of the pulp obtained (*P. cincinnata* Mast.) corroborated with some of the values found in the literature for the characterization of Caatinga passion fruit pulp cv. BRS Sertão Forte (Silva et al., 2020; Santos et al., 2021b). Silva et al. (2020) characterized the pulp of the fruit with the pH value of 2.75, SS of 8.40ºBrix, TA of 5.35% and RS of 3.15%; while Santos et al. (2021b) found pH value of 2.74, SS of 13.16ºBrix and TA of 8.41%. The differences found between these studies are acceptable and could be related to various factors, amongst which the edaphoclimatic conditions of the region of Caatinga passion fruits origin, the rainfall quantitative during cultivation, luminosity and the photosynthetic activity of the plants, and the maturation time of the fruits at the point of the processing (Freire et al., 2009; Batista et al., 2015; Kluge et al., 2015; Santos et al., 2016).

The pectin content was quantified in the majority of the trials considered (view Table 4). The pectin content of the pulp was 63.42 mg 100 g^{-1} (Table 3) and after dilution, the pectin content in the non-hydrolyzed wine must was quantified as 24.06 mg 100 g^{-1}.

Table 4 shows the decoded matrix with the treatments applied in the experimental design together with the variable response obtained (percent reduction of the pectin content). Good reproducibility of the experiment was verified from the central points (runs 15, 16 and 17) with a reduction in the pectin content in the range of 92.93% and standard deviation of ± 0.05.

Table 4. Full factorial experimental design matrix 2^3 plus axial points applied to Caatinga passion fruit wine must showed the pectin reduction response.

	Decoded Matrix			Response
Run	**Time (min.)**	**Temperature (°C)**	**Concentration (g L^{-1})**	**Pectin Reduction (%)**
1	30	45	0.02	96.26
2	120	45	0.02	96.55
3	30	55	0.02	93.52
4	120	55	0.02	91.85
5	30	45	0.05	100
6	120	45	0.05	100
7	30	55	0.05	93.93
8	120	55	0.05	93.64
9 (axial)	12	50	0.035	71.49
10 (axial)	139	50	0.035	94.60
11 (axial)	75	43	0.035	83.67
12 (axial)	75	57	0.035	87.99
13 (axial)	75	50	0.014	87.86
14 (axial)	75	50	0.056	93.56
15 (C)	75	50	0.035	92.89
16 (C)	75	50	0.035	92.93
17 (C)	75	50	0.035	92.98

Source: Authors.

On analyzing the percent pectin reductions obtained after applying the enzyme treatment, in general there was a slight variation in the responses, and thus, although the coefficients obtained were significant, the model generated and the analysis of variance at a level of confidence of 95% were not statistically significant. Therefore, the response surface for pectin was not developed due to the lack of statistical significance for this variable. However, analyzing the results punctually, the responses were considered satisfactory for the proposed study objective (accentuated reduction in the initial pectin content of the must).

Thus it can be seen that the hydrolysis efficiency reduced with increase in temperature as from 50ºC, a temperature of 45ºC being the heating condition providing the greatest efficiency (runs 1, 2, 5 and 6).

Considering these facts, any hydrolysis action time at a temperature of 45ºC and pectinase concentration of 5 g hL^{-1} resulted in the total removal of the pectin (100%) from the Caatinga passion fruit must (runs 5 and 6) as observed in Figure 1, and the tubes corresponding to trials 5 and 6 showed a color identical to that of the blank tube (containing no sample).

Figure 1. Results of the analysis of soluble pectin in Caatinga passion fruit must. Captions (from left to right): 0 = white, A = run 03, B = run 05, C = run 04, D = run 06, E = run 07.

Source: Authors.

In parallel, it can be seen that at the other temperatures, the action time and pectinase concentration influenced the hydrolysis efficiency. Hence, the shortest action time (12 min) gave the lowest efficiency of the study, resulting in the smallest pectin reduction of 71.49% (run 9). In addition, on analyzing the conditions of trial 13, although this trial applied the smallest pectinase concentration (0.014 g L^{-1}), the hydrolysis efficiency was greater than that of trial 9 due to the longer action time adopted (75 min).

Locatelli (2012) stated that in his study with pectinase, an increase in the enzyme concentration influenced the hydrolysis reaction rate up to a certain point, and one should take into consideration the fact that some factors exist that directly affect the hydrolysis process, such as the efficacy of the enzyme itself, raw material characteristics, application and efficiency of the pretreatment, presence of inhibitors and conditions of the hydrolysis process, resulting in a non-linear relationship between the enzyme dose and enzymatic conversion. Thus Haagensen (2009) affirmed that frequently, due to a variety of factors, an increase in the enzyme dose does not imply in a significant increase in the hydrolysis rate.

In addition, Taherzadeh e Karimi (2007) and Haagensen (2009) pointed out that the specific conditions of the hydrolysis process, such as the temperature and pH value, are the main factors that can affect the enzymatic activity, reducing its efficiency. The authors also stated that the action time and optimal pH factor can affect one another.

The literature reports that, in general, studies concerning the use of pectinases for industrial applications on fruits show a wide variation in optimal pH value and ideal temperature for the enzyme action, being in the range between 3 and 5.5 and between 30 and 50ºC, respectively; the ideal conditions depending on the characteristics of the enzyme to be applied in the process (Ueda et al., 1982; Jayani et al., 2005; Uenojo & Pastore, 2007; Taherzadeh & Karimi, 2007; Meneghel et al., 2016).

In their studies, Hamid & Ismail (2016) and Hamid & Ismail (2020) used an DOE to optimize the enzymatic hydrolysis process with cellulase in date seeds, testing a temperature range from 30 to 70ºC. They observed that the best conditions for the cellulase activity in their studies occurred in the range from 40 to 50ºC and defined a temperature of 45ºC as part of the optimal process conditions, a result similar to that observed in the present study with the Caatinga passion fruit must.

The authors also stated that the structures of enzymes are highly susceptible to damage caused by heat at high temperatures, and for this reason the activity of the enzymes decreases with increase in temperature as from 50ºC due to the fact that the heat provides sufficient energy to break some of the intermolecular attractions between polar molecules, such as hydrogen bonds, dipole-dipole attraction, ionic interactions and hydrophobic strength between non-polar groups inside the enzyme structure.

The observations found in the literature justify the behavior noted in the present study whereby the maximum dose of commercial pectinase used did not present the greatest efficiency, and the smallest dose used did not present the smallest efficiency, confirming that pointed out by the authors with respect to the influence of temperature on the efficiency of enzymatic hydrolysis considering different action times and pectinase doses.

Thus, the use of a factorial design method was of extreme importance in the present study, making it possible to plan and carry out the study to obtain the ideal conditions in an organized way with the minimum number of experiments, economizing time and financial resources (Silva et al., 2006; Rodrigues & Iemma, 2014).

4. Conclusion

According to the results obtained, although the application of an experimental design CCRD allowed the determination of the best conditions for the variables used (time, temperature and enzyme concentration) to obtain a total reduction of the pectin concentration present in the Caatinga passion fruit wine must, the response surface for pectin could not be obtained due to the lack of statistical significance for this variable. The ideal operational conditions of time, temperature, and commercial Endozym Pectofruit concentration recommended for the production of Caatinga Passion fruit wine are 30 min., 45ºC and 0.05 g L^{-1}, respectively. The study will serve as a base for the development of a technological production process for future studies on a pilot or industrial scale to obtain the Caatinga passion fruit wine. In addition, it is suggested to test the optimal conditions of the enzymatic treatment in other tropical fruits, for validation. The production of wine and sparkling from Caatinga passion fruit represent a potential alternative to add value to the fruit, generating more profitability to small farmers in the Caatinga Biome in Brazil.

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