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Optimization of bromelain isolation from pineapple byproducts by polysaccharide complex formation

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ABSTRACT

A simple method for bromelain extraction from industrial pineapple residues (stems and peels) was developed and optimized through factorial experimental design. The developed methodology, based on precipitation with carrageenan, represents an alternative to the use of organic solvents and inorganic salts (common industrial precipitation) and allows achieving extracts with high bromelain purity.

High recovery yield – 80–90% - of active bromelain was obtained for both crude juices (stems and peels) making possible to obtain ca. 0.3 g of bromelain from 100 g of pineapple byproducts using a low polysaccharide concentration (0.2–0.3% w/v).

1. Introduction

Tropical exotic fruit production, trade, and consumption have increased significantly on the domestic and international markets due to their attractive sensory properties and a growing recognition of its nutritional and therapeutic value (Bicas et al., 2011). Pineapple (*Ananas comosus* Merr.) is the third most popular tropical fruit and it is an important ingredient in fruit and juice-based products such as juice concentrates, jams, squash, jellies, essence, and pickles. The pineapple pulp constitutes about 30% of the whole weight of the fruit, 70% of fruit tissue is discarded as waste containing crown, peel, bottom, stem and trimmings (Chaurasiya & Hebbar, 2013; Eckenfelder, 1958). Stem alone contributes to 20% of the total waste generated by pineapple processing industry and it is usually disposed of as such.

Nowadays, pineapple waste represents a raw material that is converted into value-added products, but most of them are developed at laboratory scale. For example, cellulose and hemicellulose can be extracted from pineapple peels and used as a fertilizer or animal feed (Bartholomew, Paull, & Rohrbach, 2002). The chemical industry uses the residues for the production of methane, ethanol, citric acid and antioxidant compounds (Imandi, Bandaru, Somalanka, Bandaru, & Garapati, 2008; Nigam, 1999). Pineapple waste can be used as a source of bioactive compounds, especially proteolytic enzymes and is an alternative to waste valorization. Bromelain (BR), a sulfhydryl protease, and other cysteine proteases are well-known enzymes present in different parts of pineapple (Schieber, Stintzing, & Carle, 2001; Sunantha & Saroat, 2011). These enzymes were tested for several food industry applications, such as meat tenderization, baking industry, anti-browning agent, protein hydrolysate and alcohol production (Arshad et al., 2014). Sales of proteolytic enzymes account for over 60% of total sales of these types of biochemical products, indicating the great industrial importance of proteases (Corzo, Waliszewski, & Welti-Chanes, 2012).

The pineapple plant contains at least five distinct cysteine proteases belonging to the papain family. The major protease present in pineapple stem (heart and cylinder of the pineapple) is stem BR (EC 3.4.22.32) and the other minor protease include Ananain (EC 3.4.22.31), Comosain and SBA (acidic stem BR) (Maurer, 2001). Fruit BR (EC 3.4.22.33) is the major protease in the pulp (Yamada, Takahashi, & Murachi, 1976). Pineapple crude extract is a mixture of different cysteine proteases with similar amino acid sequences and different enzymatic activities. Stem BR has been generally obtained

Abbreviations: BR, bromelain; Carr, carrageenan; LNPE, N-α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester (Z-L-Lys-ONp)

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from the juice of pineapple waste (mainly stems) through precipitation with organic solvents (e.g., acetone and methanol) or ultrafiltration (Heinicke, 1961).

Polyelectrolyte precipitation is a friendly and efficient separation technique that is gaining importance towards the concentration and purification of proteins (Schmitt, Aberkane, & Sanchez, 2009). Polyelectrolytes can interact with proteins forming soluble or insoluble complexes. By changing medium conditions, such as pH values or ionic strength, the protein can be released, keeping its structure as well as its biological activity (Valetti, Lombardi, Boeris, & Picó, 2012). This natural interaction can be used to form interest molecules complexes in order to separate them from the medium. Carrageenan (Carr) is a collective term for non-toxic hydrophilic linear sulfated galactans extracted from certain species of red seaweed (*Rhodophyceae*). Commercial Carr has an average molecular weight (MW) ranging from 400 to 600 kDa, and a minimum of 100 kDa (van de Velde & De Ruiter, 2002).

Until now, every methodology applied to enzyme precipitation uses organic solvents or inorganic salts. These processes show low extraction yield as well as enzymes activity loss (Valetti et al., 2012). On the other hand, chromatography columns are used in order to separate purified molecules of interest, but this kind of methodology represents high costs and low amount of product obtained (Coelho, Silveira, Junior, & Tambourgi, 2013; Gautam, Mishra, Dash, Goyal, & Rath, 2010). Even though polyelectrolyte precipitation was evaluated at a fundamental level, it has never been applied for the purification of BR from pineapple residues and never optimized using the appropriate statistical tools. A detailed literature review shows that polyelectrolyte precipitation was only successfully tested on a model system using pure enzymes (Fabian, Huynh, & Ju, 2010; Schmitt et al., 2009).

Thus, the objective of this work is to develop an affordable new green process based on the use of natural polymers for the precipitation and purification of BR from pineapple residues, namely stems and peels.

2. Materials and methods

2.1. Raw materials

Fresh pineapples at ¾ stage maturity (*Ananas comosus* Merr.) were purchased from Costa Rica and exported to Portugal. The pineapple fruit was processed automatically detaching the crown and stem, and peeling off the skin, in a commercial fruit processing. The residue parts were frozen at −20 °C for a maximum period of 90 days until further use.

2.2. Chemical

Standard BR from pineapple stem, ι-carrageenan, N-α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester (Z-L-Lys-ONp) (LNPE), L-cysteine and all the other reagents of analytical quality were purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

2.3. Preparation of crude juice

The pineapple (stem and peels) were reduced to juice, using a juice machine (model: MES1020 of 380 W, Bosch) that separates major solids from the liquid. The crude juice was centrifuged at 7370 *g* for 10 min, at 4 °C. The supernatant was centrifuged again. The remaining supernatant was evaluated for its BR activity. All parts were frozen at −20 °C. To check eventual loss of protein structural integrity during storage, measurement of enzyme activity was carried out before using.

2.4. Determination of BR activity

Bromelain activity was determined using the substrate LNPE (Heinrikson, 1976) and performed exactly as described by Campos et al. (2017). Briefly, the substrate was used at a final concentration of 0.23 mM in 30 mM sodium acetate buffer, pH 4.6, supplemented with 100 mM KCl and 1.0 mM of L-Cysteine. The extent of the enzymatic reaction, represented by the release of *p*-nitrophenol, was measured spectrophotometrically for 5 min at 340 nm, 25 °C and with continuous stirring. One unit of enzymatic activity is defined as the amount of BR that releases 1.0 mol of *p*-nitrophenol from LNPE in 1 min under the experimental conditions. The method was adapted to microwell plate use and the final reaction volume was 300 μL. Reaction mixture with no enzyme was used as control. The activities were calculated from the slope (m) of the initial linear portion of absorbance vs. time curve. The BR concentration was calculated using a standard calibration curve, using the same method. The calibration curve was performed between 18 and 75 $\mu g\ mL^{-1}$ of standard BR and a linear equation was obtained through BR concentration vs BR activity (m) slope. Thus, is possible to calculate BR concentration in solution.

2.5. Determination of total protein concentration

Total protein was determined using the bicinchoninic acid assay (BCA) (Brown, Jarvis, & Hyland, 1989; Walker, 2009). A fresh standard working reagent (SWR) was prepared mixing 100 vol of reagent A (bicinchoninic acid solution; Sigma–Aldrich, St. Louis, Missouri, USA) with 2 vol of reagent B ($CuSO_4$ solution 4% (w/v) prepared from $CuSO_4{\cdot}5H_2O$; Sigma–Aldrich, St. Louis, Missouri, USA). A volume of 50 μL of protein solution (maximum concentration of 1 $mg\ mL^{-1}$) was added to 1 mL of SWR. The tubes were incubated at 37 °C, for 30 min. After cooling down to room temperature, the absorbance was measured at 562 nm using a cell with a 1 cm path length. The calibration curve was performed using dilutions of bovine serum albumin standard solution).

2.6. Turbidimetric titration curves with carr

The formation of the insoluble BR-Carr complex was monitored by means of turbidimetric titration. A fixed concentration of each crude juice (stem and peel; 65 and 80 $mg\ mL^{-1}$ respectively) was titrated at 25 °C using 0.05–0.5% (w/v) Carr solution as titrant. The absorbance of the solution was measured at 420 nm to follow the BR-Carr complex formation and plotted vs. the total Carr concentration in the microwell plate. Absorbance was measured using a MicroPlate Reader FLUOstar OPTIMA (VGM LabTech, Telford, UK) with constant agitation in a thermostatted environment (Heinrikson, 1976).

2.7. SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using discontinuous gel, 13% (w/v) polyacrylamide for resolution gel and 4% (w/v) polyacrylamide for a stacking gel. Aliquots of pineapple stem crude juice, supernatant and redissolved precipitate were analyzed, using a vertical system. The running time was 120 min and the intensity was constant at 25 mA for the resolving gel. Proteins were stained with Coomassie brilliant blue (results were presented as supplementary material).

2.8. Analysis by size exclusion chromatography

The molecular weight distribution of BR was studied by gel filtration chromatography. The column was operated at a flow rate of 0.5 $mL\ min^{-1}$ with 0.025 M phosphate buffer (pH 7) containing 0.15 M NaCl and 0.2 $g\ L^{-1}$ NaN_3. Standard proteins with known molecular weights (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotinin, 6.5 kDa) were used to establish the MW standard curve. AKTA pure 25 L system, from GE Healthcare Life Sciences (Freiburg, Germany), was used with a configuration of two pumps with pressure control for column protection, a gel filtration column prepacked with Superdex® 200 10/300 GL connected in series

with a column Superdex Peptide 10/300 GL (GE Healthcare Life Sciences, Freiburg, Germany), and an UV multiwavelength detection monitor U9-L, at a fixed wavelength of 280 nm. The software used to evaluate samples was UNICORN 7.0.

2.9. Quantification by high-pressure liquid chromatography

Separation method used was adapted from Ee, Zhao, Rehman, and Agboola (2009) and was carried out for protein quantification. Two different mobile phases were applied, mobile phase A – water and acid trifluoroacetic (TFA) (99.9:0.1, v/v) - and mobile phase B – acetonitrile and TFA (99.9:0.1, v/v), – under the following conditions: gradient elution starts at 100% mobile phase A and ends at 45% after 45 min, at a continuous flow of 0.5 mL min^{-1}. Between 45 and 50 min the mobile phase A returns to 100% and remains at this percentage for 2 min (until 52 min). Detection was performed at a wavelength of 280 nm, peaks were analyzed and quantified using a calibration curve of pure protein (HPLC gradient) comparing retention time and spectra. The chromatographic analysis was performed using a Waters e2695 separations module system interfaced with a Photodiode array UV/Vis detector (PDA 190–600 nm). Separation was performed in a reverse phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm; Dartford, UK). Data acquisition and analysis were accomplished using Software Empower 3. Three independent analysis were performed for each experiment.

2.10. Enzyme proteolytic assay

The protease activities were assayed using azocasein as substrate and adapted by Leighton, Doi, Warren, and Kelln (1973) to small volumes, and resulting reaction was measured spectrophotometrically at 450 nm. In duplicate, using microcentrifuge tubes, 50 µl of 1% (w/v) azocasein (Sigma-Aldrich), prepared in 0.2 M Tris-HCl pH 9, was incubated with 30 µl of crude juice for 60 min at 25 °C 240 µl of 10% (w/v) trichloroacetic acid (TCA) was then added to stop the reaction. After 15 min at rest, the tubes were centrifuged for 5 min at 8000 g 70 µl of the supernatant was then added to 130 µl of 1 M NaOH and the absorbance of the mixture was measured. A blank was prepared in the same way, replacing the crude juice with 0.9% (w/v) of NaCl. Previous experiments showed that under the conditions described above, the first 0 min of the reaction follows first order kinetics. One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze azocasein resulting in an increase of 0.001 units of absorbance per minute.

2.11. Experimental statistical design

A factorial experimental design was applied to determine the best combination of variables to obtain the highest amounts of BR in precipitates. The definition of independent variables (responses) and their appropriate ranges were based on a 2-level complete factorial design. The initial factorial design considered four independent variables described elsewhere (Schmitt et al., 2009) as influents in the protein-polysaccharide complexes formation and three dependents variables (experimental factors). Responses that presented no significant effect ($P > 0.05$) were eliminated from the following statistical design. The experimental design and calculation of the predict data were carried out with StatGraphics® Centurion XVI (statistical software from StatPoint Tecnologies, Inc.).

2.11.1. Statistical model design for standard BR

Central Composite Design (CCD) with star points was applied to determine the variables combination which influenced the complex formation between BR and Carr. Three responses (BR concentration, Carr concentration and pH value) and three experimental factors were used. The responses were studied at three levels coded as −1 (lowest level), 0 (central level), +1 (highest level). The complete design consisted of 16 experimental trials, including two repetitions of the central point. Each system was evaluated in triplicate. Responses were adjusted to the following second-order polynomial model (equation (1)):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{1,2} X_1 X_2 + \beta_{1,3} X_1 X_3 + \beta_{2,3} X_2 X_3 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2 + \varepsilon \quad (1)$$

where Y is the measured response; β_0 is the intercept (constant); β_1 to $\beta_{3,3}$, are the coefficients associated with linear, quadratic and interaction effects, respectively, of variables X_1, X_2 and X_3, respectively and ε is the (random) error. The models examine the effect of each independent variable as well as, all interactions between them on a particular response. Table 1 lists the coded and un-coded levels of the independent variables. Turbidimetry, BR activity and total protein after precipitation and re-dissolution of the complex were the responses evaluated. A predictive model was used to graphically represent the systems.

Table 1
Independent variables and levels used for response surface design.

CCD using standard BR	**Variables**	**Level**		
		−1	0	1
	BR concentration (mg mL^{-1})	1.0	3.0	5.0
	Carr concentration (µg mL^{-1})	50.0	150.0	250.0
	pH of final solution	3.0	5.0	7.0
BBD using pineapple stem crude juice	**Variables**	**Level**		
		−1	0	1
	Stem crude juice concentration (mg mL^{-1})	45.0	55.0	65.0
	Carr concentration (µg mL^{-1})	50.0	175.0	300.0
	pH value of final solution	4.5	7.0	9.5
BBD using pineapple peel crude juice	**Variables**	**Level**		
		−1	0	1
	Peel crude juice concentration (mg mL^{-1})	60.0	70.0	80.0
	Carr concentration (µg mL^{-1})	50.0	125.0	200.0
	pH value of final solution	4.5	7.0	9.5

2.11.2. Statistical model design for BR crude juices

Box-Behnken Design (BBD) was applied to determine the combination of variables which would give the optimum complex formation between protein and polysaccharides, for each crude juice. The responses evaluated are described in Section 2.9.1. The design consisted of 15 experimental trials, including two repetitions of the central point. Each of the 15 systems was performed with three repetitions and evaluated in triplicate. Responses were adjusted to a second-order polynomial model (equation (1)).

Finally, a multi-criterion optimization based on the Derringer's desirability function was applied (Suich & Derringer, 1980) to the results of the experimental design, expressing the desirability of each response value on a scale of 0–1.

3. Results and discussion

3.1. Gel filtration chromatography

Fig. 1 shows the MW distribution of standard stem BR at 100 mg mL^{-1}, as well as pineapple natural juices (stem and peel). For standard stem BR, two major peaks were observed (peak 1 at 17.7 mL and peak 2 at 20.5 mL). The elution values represented a molecular size of ± 31 kDa (peak 1), which was compatible with fruit BR and a molecular size of ± 23.8 kDa compatible with stem BR. These results are in accordance with the ones obtained by de Lencastre Novaes et al. (2016). Both crude juices presented one major peak at ca. 20.0 mL (± 25 kDa), which were in the same range of stem BR, indicating a high concentration of this enzyme in both crude juices.

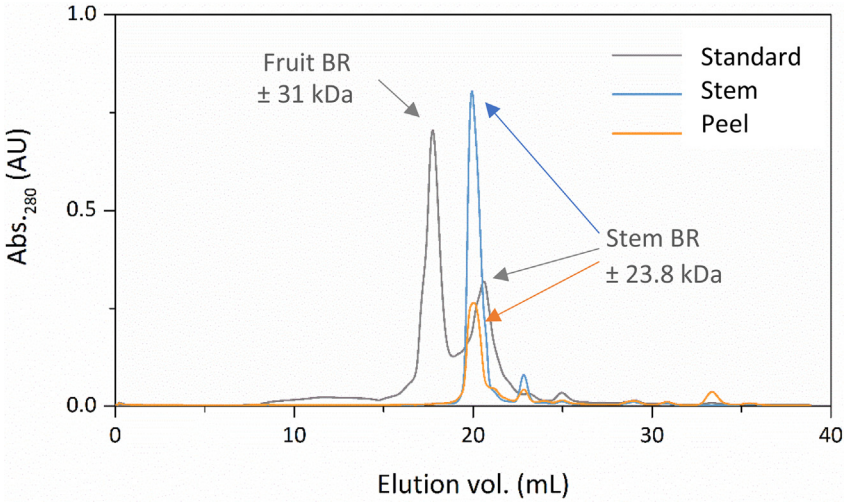

Fig. 1. Typical size exclusion chromatograms obtain through FPLC using standard BR and the two different pineapple byproducts crude juices (stems and peels). Fruit BR presented a molecular weight of ± 31 kDa and Stem BR of ± 23.8 kDa.

3.2. Complex formation phase diagrams of carr-pineapple crude juices

Bromelain precipitation was studied using pineapple byproduct crude juices (stem and peels). Thus, a constant concentration of each crude juice was incubated with increasing Carr concentration, at pH 4.6. Insoluble complexes were separated by centrifugation and redissolved in Tris-HCl buffer, pH 8.2, with the addition of 500 mM of NaCl as described before by Campos et al. (2017). Bromelain enzymatic activity was determined in supernatant and precipitate, and results are shown in Fig. 2. For stem crude juice and in order to recover 50% of available BR present in the precipitate, a low concentration of Carr (0.05%, w/v) was used. Also, in average, ca. 90% of the activity was recovered in the solubilized precipitate for 0.3% of Carr (w/v), while 10% of the activity remained in the supernatant, confirming that the equilibrium was displaced to a large extent to the insoluble complex formation. In the case of peel crude juices, to achieve a 50% recovery of the available BR, a higher Carr (0.1%, w/v) concentration was needed to obtain insoluble complex. However, only 0.2% (w/v) of Carr was needed to transfer ≥80% of available BR to the precipitate, while ca. 10% of activity remained in the supernatant. When testing higher concentrations of Carr (≥0.25%, w/v) a reduction of activity on the precipitate was observed. Other authors (Valetti et al., 2012) reported the similar behavior when extracting enzymes from animal samples by the high concentration or excessive use of polyelectrolytes in a solution, leading to proteases activity loss.

This is the first time that polyelectrolytes systems are successfully applied to fruit crude juices for BR recovery and no other result on the literature is comparable.

3.3. Experimental design for limit definition of BR complex formation

Considering a previous work described by Campos et al. (2017), where the separation mechanism of BR by application of a polysaccharide was enlightened and taking into account the possible future application in the industry, the experimental design was used to facilitate a scale-up. As explained before, the pineapple residues (stem and peels) processed in a juice machine, and different parts were separated and stored.

Response analysis was initially evaluated for a standard BR as it made easier to evaluate the precipitation process. Crude juices (stem and peels) contain several other compounds such as polysaccharides, simple sugars, fibers and other proteins that could have a direct effect on complex formation (Schmitt et al., 2009). The experimental conditions to use in the experimental design were selected by investigating

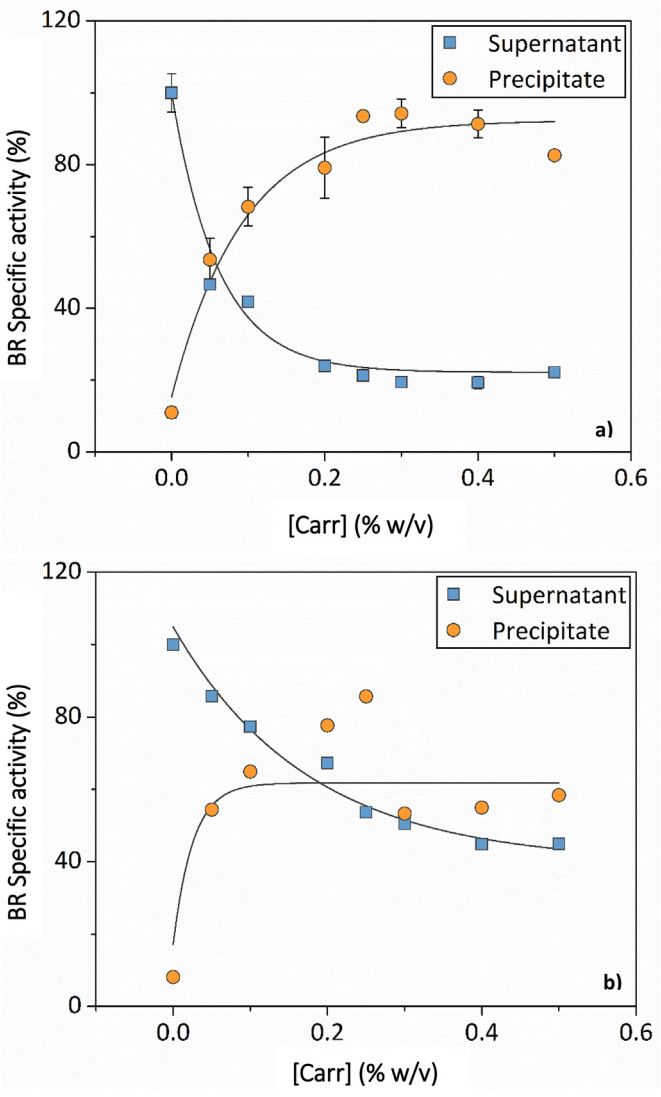

Fig. 2. Recovery of BR and activity measurement in the precipitate and in the supernatant, at different initial concentrations of Carr. Medium: pineapple byproducts crudes juices (stems (a), and peels (b)), pH in solution of 4.3. Enzyme activity was measured at pH 5.1.

the factors that influence the BR-Carr complex formation. In order to simplify the final model (response surface design), single-factor experiments were performed as well as a complete factorial design to determine which independent variables had a significant effect and which did not (data not shown). Protein (0–10 mg mL^{-1}), and polysaccharide concentration (0–500 μg mL^{-1}), pH (3.0–9.6), and time of contact between protein and polysaccharide (0–120 min) were considered as responses, finding that the time of contact did not significantly influence the model.

Based on the data obtained for standard BR, the limits to be used for BR recovery from crude juices were established and models developed. Limits previously tested were narrowed as described in Table 1.

3.4. Surface model design for standard BR

For the CCD, three responses were evaluated: turbidity by absorbance at 420 nm, relative enzymatic activity (as described in Section 2.4) and total proteins (by BCA method). These variables allowed to evaluate the transfer of the free protein in solution (supernatant) to a complexed protein in the precipitate.

After the statistical analysis of variance of the experimental design, the statistical model was adjusted to the responses with R^2 that vary between 95.42% and 99.99% (Table 2).

The results showed that BR precipitation was more affected by the BR concentration (X_A), followed by pH value (X_C) and then Carr concentration (X_B). It was evident that most of the interaction parameters were not significant, except for X_AX_C. The predict response Y for the BR precipitation could be expressed by the following second-order polynomial equation in term of coded values:

$$Y(Turbidimetry) = 1.10 + 0.37X_A + 0.17X_B - 0.01X_C + 0.18X_AX_B + 0.13X_AX_C + 0.05X_BX_C - 0.22X_A^2 - 0.18X_B^2 - 0.05X_C^2$$

$$Y(Relative\ activity) = 33.46 - 98.59X_A - 2.92X_B + 29.77X_C + 29.15X_AX_B + 110.52X_AX_C - 35.25X_A^2 - 64.97X_C^2$$

Table 2
Range and codification criterion for independent variables in the CCD using standard BR. Desirability values for an optimized model for isolation of BR.

Exp.	Coded values			Natural values			Turb	Responses	TP
	X_A	X_B	X_C	X_A	X_B	X_C		RelAct	
1	0	1.5	0	3	300	5	1.005	327	0
2	0	0	0	3	150	5	1.104	337	165
3	−1	1	−1	1	250	3	0.434	187	124
4	0	0	0	3	150	5	1.091	333	187
5	−1	−1	1	1	50	7	0.178	69	0
6	1	−1	1	5	50	7	0.653	0	6
7	−1	1	1	1	250	7	0.165	41	0
8	1	1	−1	5	250	3	1.090	245	238
9	0	−1.5	0	3	0	5	0.000	0	0
10	1	−1	−1	5	50	3	0.599	141	118
11	0	0	−1.7	3	150	1.6	0.000	0	310
12	−1	−1	−1	1	50	3	0.465	245	52
13	−1.5	0	0	0	150	5	0.000	0	0
14	1	1	1	5	250	7	0.000	0	0
15	0	0	1.7	3	150	8.4	0.973	197	127
16	1.7	0	0	6.4	150	5	1.139	400	269

Variables	Factor values			Responses	Values
	Optimum	Low	High		Optimum
X_A (mg mL^{-1})	**3.74**	1.0	5.0	**Turbidity**	1.139
X_B (μg mL^{-1})	**132.0**	50.0	250.0	**Enzymatic activity**	400.2
X_C	**6.44**	3.0	7.0	**Total proteins**	159.52

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH. Turbidimetry (Turb), relative activity (RelAct) and total protein (TP).

$$Y(Total\ protein) = 175.45 + 65.28X_A + 31.14X_B - 44.88X_C + 16.99X_AX_B - 13.11X_BX_C - 20.28X_A^2 - 66.71X_B^2 + 12.04X_C^2 \quad (2)$$

Through the analysis of the equations and consequently the variance, the statistical significance of each effect was verified. In the analysis of turbidity, it was possible to verify that only pH effect was not significant, proceeding to its removal from the analysis. It was possible to conclude that 8 of the 9 effects studied significantly influenced the complex ($P < 0.050$) formation. The BR and Carr concentrations and the interaction between these factors presented a positive effect in turbidity formation, which means that the response is directly proportional with the tested concentrations. On the other hand, these factors quadratic coefficients showed a negative effect, indicating that there is an increase in turbidity at intermediate values. Although pH did not show a significant effect, the effects of its interaction with BR and Carr concentration were significant. Thus, removing the non-significant effects, the model was adequate, showing a good response to variability ($R^2 = 98.10\%$) and an adequate fit ($P > 0.050$).

For the relative enzymatic activity, it was possible to verify that two effects were not significant (quadratic coefficient of polysaccharide concentration and its interaction with pH) and, therefore, they were removed from the model. After evaluation of the adjusted model, the linear coefficient of X_B (Carr concentration) was also removed because it did not present a significant effect when concerning the enzymatic activity, which can be corroborated by the results presented in equation (2).

A similar behavior was found when responses were compared, relative enzymatic activity and turbidity since the quadratics coefficients presented a negative effect, meaning that lower enzymatic activity was not achieved for the extreme tested values, but somewhere in the middle. On the other hand, the remaining linear coefficients (BR concentration and pH) as well as their interaction, showed a positive effect on the enzymatic activity, which means that the enzymatic activity was directly correlated with the increasing presence of BR and therefore pH changes; higher concentration of BR and pH values, higher relative enzymatic activity on the precipitate. The final adjusted model showed a high response to variability as can be seen by the followed values $R^2 = 98.49\%$ and a significant fit ($P > 0.050$).

For total proteins, none of the factors had a significant effect. Thus, the model was adjusted to best fit, removing from the analysis one non-significant effect (interaction coefficient between BR concentration and pH). The *lack of fit* values demonstrates that the model was appropriate to the observed data.

Through the statistical analysis, it was possible to determine a model for complex formation between standard protein and the tested polysaccharide (BR-Carr), with the final objective of BR purification. The desirability was used to estimate the combination of experimental factors, that simultaneously optimizes the responses. Several features were established in this specific model – purification of BR (passage of BR from supernatant to a precipitate): the maximization of turbidity in the solution; maximization of enzymatic activity in the precipitate; and minimization of the total proteins in the precipitate. The mathematical model allowed the calculation of the optimal value of each factor, maximizing the biological precipitation process of BR, as shown in Table 2.

3.5. Experimental models design applied to pineapple crude juices

To create a model for each crude juice, the BR was quantified by HPLC method, as described above (BR from Sigma-Aldrich, was used as standard). The stem and peel crude juices presented a BR concentration of ca. 70 mg mL^{-1} and ca. 85 mg mL^{-1}, respectively. For the design of the extraction model, a high concentration of crude juice was established to mimetize a possible application at industrial scale. The concentrations were adjusted for each model, namely, stem crude juice between 45 and 65 mg mL^{-1} and peel crude juice between 60 and

80 mg mL^{-1}.

3.6. Stem crude juice

Four responses were evaluated: turbidity; relative enzymatic activity in supernatant; total proteins in supernatant; and specific enzymatic activity in the supernatant (calculated by equation (3)).

$$Specific\ enzimatic\ activity = \frac{Relative\ enzimatic\ activity\ in\ solution}{Total\ protein\ in\ solution} \quad (3)$$

The variance of the observed data was explained by the applied model since it presented an R^2 that varied between 81.60 and 95.65%.

Bromelain precipitation was mainly affected by the variation in enzyme concentration (X_A). The pH value (X_C) and Carr concentration (X_B) also had significant effects but at a smaller extension. It was evident that most of the interaction parameters were not significant, except X_AX_C. The predict response Y for the BR precipitation could be expressed by the following second-order polynomial equation in term of coded values:

$$\begin{aligned} Y(Turbidimetry) &= 0.96 + 0.24X_A - 0.06X_C + 0.04X_AX_C - 0.06X_B^2 \\ Y(Relative\ activity) &= 10.65 - 4.19X_A + 6.27X_C - 6.73X_AX_B - 8.17X_AX_C \\ &\quad + 5.59X_B^2 \\ Y(Total\ protein) &= 4659.02 + 760.44X_A \\ Y(Specific\ activity) &= 0.002 - 0.001X_A + 0.001X_C - 0.002X_AX_B \\ &\quad - 0.002X_AX_C + 0.001X_A^2 + 0.001X_B^2 \end{aligned} \quad (4)$$

An analysis of variance was performed to determine which factors significantly affected the BR precipitation by complex formation (Table 4). The statistical analysis allowed the search for the significance of each effect (independent variables and their interactions). When studying turbidity, it was possible to verify that only 4 effects (two linear coefficients X_A and X_C, one interaction coefficient - X_AX_C and one quadratic coefficient of - X_B^2) significantly influenced the complex formation ($P < 0.050$) (Fig. 3). As can be seen in equation (4), the coefficient X_A has a positive effect, which means that has higher BR concentration in solution and therefore higher turbidity. On the other hand, the other linear coefficient X_C has a negative effect which means that the increase in pH values decrease the solution turbidity. When studying the interactions between these variables a positive effect was observed, meaning the formation of turbidity was directly proportional to enzymatic concentration increase and inversely proportional to pH (Fig. 4, response surface graphic). The quadratic coefficient of X_B showed a negative response, which means that the constant increase of polysaccharide concentration in solution did not affect, in a linear manner, the turbidity formation, so the higher value (maximum) was obtained elsewhere. The values of the adjusted model are described in Table 4a, indicating that the model fits the observed data.

Table 4a
Analysis of variance (ANOVA) of Turbidimetry using crude juice from pineapple stem for the results of the BBD (R^2 = 96.36, $R^2_{adjusted}$ = 95.65).

Factor	SS[a]	DF[b]	MS[c]	F	p
X_A	1.302	1	1.302	377.6	0.0000
X_C	0.083	1	0.083	24.2	0.0002
X_AX_C	0.022	1	0.022	6.3	0.0241
X_B^2	0.040	1	0.040	11.7	0.0038
Blocks	0.658	2	0.329	95.5	0.0000
Lack of fit	0.047	20	0.002	0.68	0.7927

Abbreviations: BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

At the statistical analysis of variance for the response – relative enzymatic activity, the model showed that 3 effects were not significant (X_B, X_B^2 and X_BX_C) being removed from the model (Fig. 3). Thus, six effects presented a $P < 0.050$ indicating that they were significant. The coefficients involving X_A, the linear and interaction coefficients (X_AX_B

Table 3
Range and codification criterion for independent variables in the BBD using crude juice from pineapple stem. Desirability values for an optimized model for isolation of BR.

Exp.	Coded values			Natural values			Turb	RelAct	TP	SpeAct
	X_A	X_B	X_C	X_A	X_B	X_C				
1	1	0	−1	65	125	4.5	1.23 ± 0.23	5.5 ± 0.71	5320 ± 428	0.0009 ± 0.0001
2	0	−1	1	55	50	9.5	0.96 ± 0.17	19.0 ± 1.41	4883 ± 757	0.0040 ± 0.0009
3	0	1	−1	55	200	4.5	0.97 ± 0.18	7.5 ± 0.71	4457 ± 470	0.0015 ± 0.0004
4	0	0	0	55	125	7	0.97 ± 0.14	7.7 ± 1.53	4359 ± 224	0.0016 ± 0.0003
5	1	0	1	65	125	9.5	1.18 ± 0.18	8.0 ± 2.00	5260 ± 173	0.0015 ± 0.0003
6	−1	0	−1	45	125	4.5	0.83 ± 0.11	4.3 ± 2.08	3930 ± 175	0.0011 ± 0.0006
7	−1	0	1	45	125	9.5	0.62 ± 0.15	36.7 ± 3.06	3876 ± 373	0.0087 ± 0.0010
8	1	−1	0	65	50	7	1.18 ± 0.15	22.0 ± 8.49	5488 ± 55	0.0039 ± 0.0018
9	−1	−1	0	45	50	7	0.68 ± 0.20	11.0 ± 1.41	3954 ± 146	0.0026 ± 0.0000
10	0	−1	−1	55	50	4.5	0.97 ± 0.14	15.3 ± 1.16	4855 ± 175	0.0030 ± 0.0002
11	0	1	1	55	200	9.5	0.85 ± 0.16	21.7 ± 2.52	4877 ± 120	0.0042 ± 0.0002
12	1	1	0	65	200	7	1.12 ± 0.18	12.0 ± 2.83	5511 ± 356	0.0022 ± 0.0007
13	0	0	0	55	125	7	1.00 ± 0.17	9.7 ± 2.08	4886 ± 523	0.0020 ± 0.0006
14	−1	1	0	45	200	7	0.61 ± 0.11	26.0 ± 2.83	3737 ± 130	0.0066 ± 0.0016
15	0	0	0	55	200	7	0.95 ± 0.11	12.0 ± 2.83	4702 ± 385	0.0020 ± 0.0000

Variables	Factor values			Responses	Values
	Optimum	Low	High		Optimum
X_A (mg mL^{-1})	**52.7**	45.0	65.0	**Turb**	0.9765
X_B (μg mL^{-1})	**166.5**	50.0	300.0	**RelAct**	3.54
X_C	**4.5**	4.5	9.5	**TP**	4482
				SpeAct	0.00059

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH. Turbidimetry (Turb), relative activity (RelAct), total protein (TP) and specific activity (SpeAct).

Table 4b
Analysis of variance (ANOVA) of Relative Activity using crude juice from pineapple stem for the results of the BBD (R^2 = 86.78, $R^2_{adjusted}$ = 84.70).

Factor	SS[a]	DF[b]	MS[c]	F	*p*
X_A	329.2	1	329.2	24.6	0.0008
X_C	812.2	1	812.2	60.8	0.0000
X_AX_B	353.8	1	353.8	26.5	0.0006
X_AX_C	714.0	1	714.0	53.4	0.0000
X_A^2	88.0	1	88.0	6.6	0.0304
X_B^2	277.8	1	277.8	20.8	0.0014
Blocks	85.4	2	42.7	95.5	0.0895
Lack of fit	364.4	19	19.2	0.68	0.2961
Pure error	120.3	9	13.4		
Total SS	3168.9	36			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

and X_AX_C) showed a negative effect on the equation, which means that the increased concentration of protein, decreased the enzymatic activity in solution, a higher amount of BR was passing from supernatant to precipitate and therefore higher complex formation was produced. The statistically significant quadratic coefficients were studied (X_B^2) and presented a positive effect on the response, which means that higher values of relative enzymatic activity were achieved near the minimum and/or maximum values of protein and polysaccharide in solution. The linear coefficient X_C, was statically significant and showed a positive effect on the relative enzymatic activity, higher pH value and higher enzymatic activity on a solution. These results are in accordance with those obtained for turbidity. The change (or moving) of BR from supernatant to precipitant was favorable for lower pH values. The values of adjustability are described in Table 4b, and these demonstrate that the model is adequate for the data observed.

The analysis of variance of the response – total protein showed that only one principal effect (X_A; BR concentration in supernatant) was significant (P < 0.050) (Fig. 3). Thus, for this response, only BR presence affected directly the total protein in solution, as expected. The model showed an adjustability value of 0.4886 and an R^2 of 81.60% (Table 4c), indicating that it is adequate to explain the observed data.

Considering the response – specific enzymatic activity (calculated through equation (4)), which evaluates the amount of remaining active enzyme in the supernatant, the statistical analysis showed that three of the nine effects were not statistically significant (P > 0.05). Thus, X_B, X_BX_C and X_C^2 were removed from the final model (Fig. 3). Three factors showed a positive effect on the specific enzymatic activity, the linear coefficient X_C (pH) and the two quadratic coefficients X_A^2 (BR concentration) and X_B^2 (Carr concentration). These results show that pH increasing allowed the increasing of enzymatic activity on supernatant, thus the lower pH tested allowed the formation of a complex between BR and Carr, and therefore leading to the BR precipitation. Through

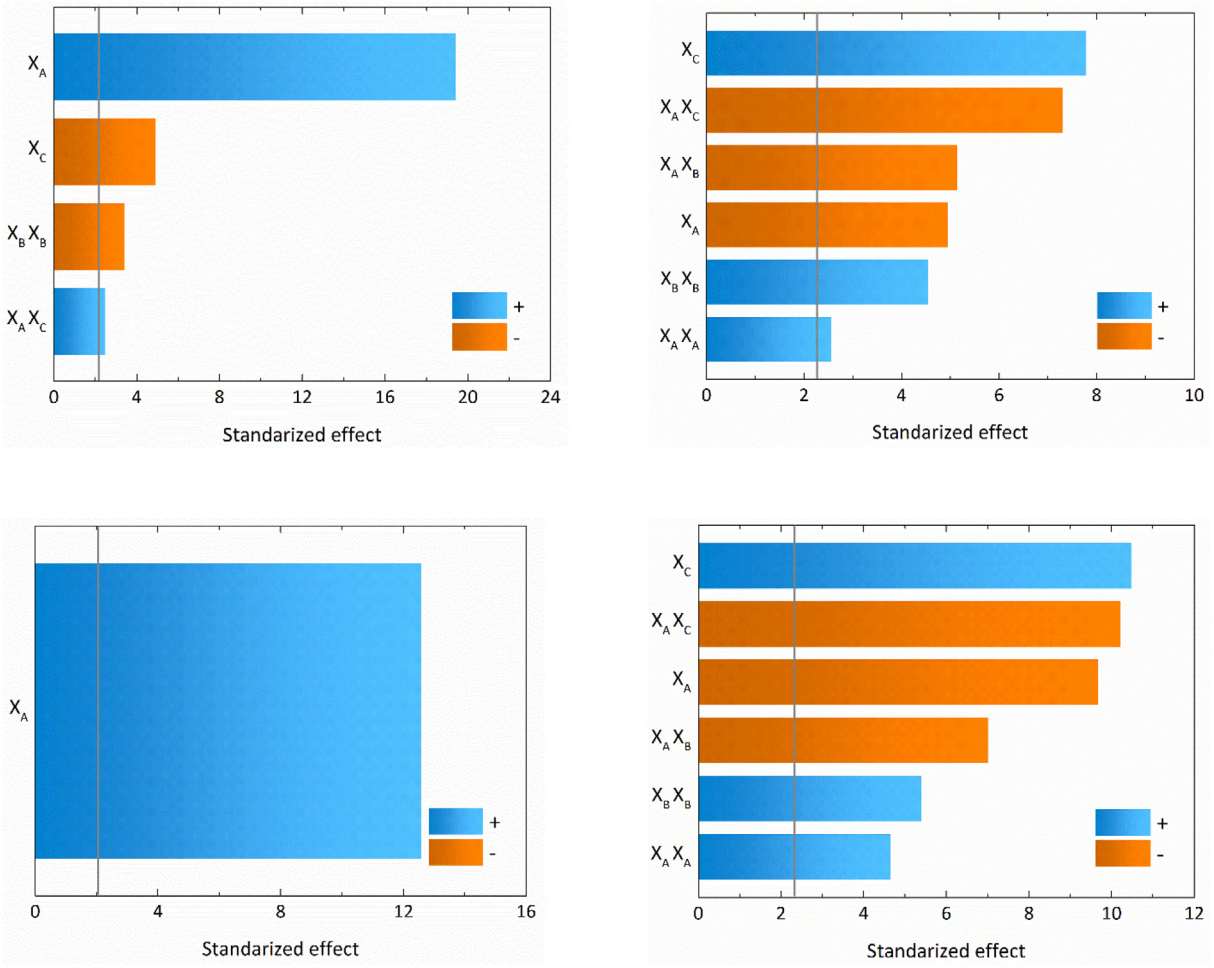

Fig. 3. Standardized Pareto charts encompassing the effect of each independent variable (i.e., (X_A) - BR (mg mL^{-1}), (X_B) - Carr (μg mL^{-1}) and (X_C) - pH, divided by its standard error, pertaining to four responses (i.e., turbidity, relative activity of BR (mg g^{-1}), total protein (mg g^{-1}) and specific activity of BR), obtained from pineapple stems crude juices. The vertical line in each chart represents the 10% significance level.

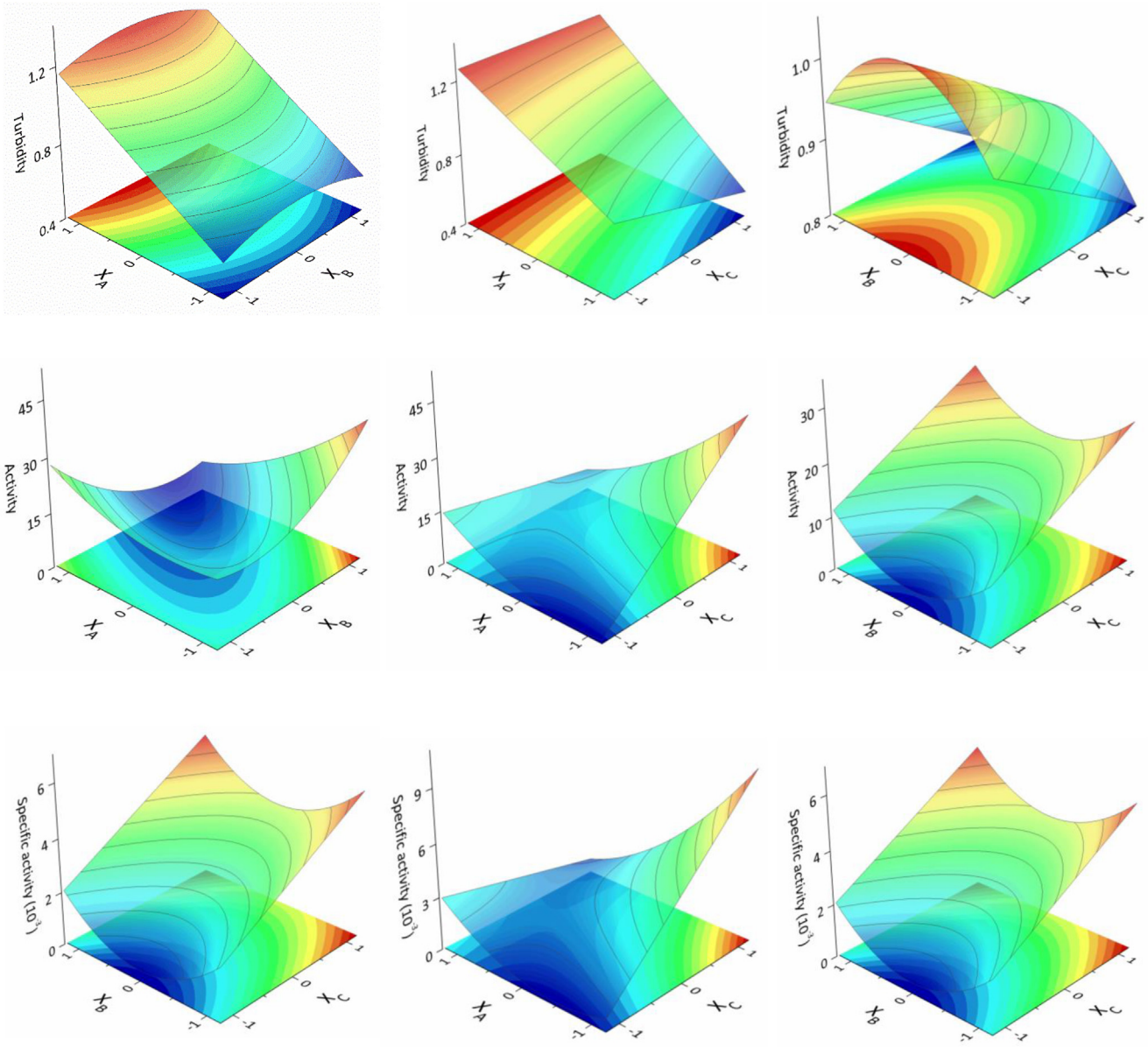

Fig. 4. Response surfaces corresponding to the combined effect of (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH. Turbidimetry (Turb), on the maximum passage of BR towards the precipitate through the formation of complex with Carr, according to equation (4). Four responses were evaluated (i.e., turbidity, relative activity of BR (mg g^{-1}), total protein (mg g^{-1}) and specific activity of BR), obtained from pineapple stems crude juices.

Table 4c
Analysis of variance (ANOVA) of Total Protein using crude juice from pineapple stem for the results of the BBD (R^2 = 84.32, $R^2_{adjusted}$ = 81.60).

Factor	SS[a]	DF[b]	MS[c]	F	*p*
X_A	0.00000014	1	0.00000014	158.43	0.0008
Blocks	706153.0	2	353076.0	4.03	0.0272
Lack of fit	397090.0	5	79417.9	0.91	0.4886
Pure error	0.00000289	33	87599.0		
Total SS	0.00000018	41			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

quadratic positive coefficients, it was possible to understand that the minimum and maximum values tested for BR and Carr concentrations allowed a higher concentration of protein in solution. The minimally tested concentrations did not allow the formation of complex between protein and polysaccharide. On the other side, as reported before in this work, other authors (Valetti et al., 2012) reported the same behavior for chymotrypsin from animal source, where polyelectrolytes at a very high concentration or in excess in a solution may lead to loss of proteases activity. Thus, the optimum conditions for complex formation were the intermediate concentrations.

Three factors presented a negative effect on this response, the linear coefficient X_A (BR concentration) and its interactions coefficients X_AX_B (between BR and Carr) and X_AX_C (between BR concentration and pH). Linear coefficient showed that higher concentration of BR lead to lower enzymatic activity in solution, indicating that a higher amount of protein was recovered towards precipitate. The interactions showed a high level of correlation between effects, hampering to describe a conclusion from the negative interactions. Thus, the model showed an

Table 4d
Analysis of variance (ANOVA) of Specific Activity using crude juice from pineapple stem for the results of the BBD (R^2 = 89.00, $R^2_{adjusted}$ = 88.12).

Factor	SS[a]	DF[b]	MS[c]	F	*p*
X_A	0.0000359	1	0.0000359	93.6	0.0000
X_C	0.0000418	1	0.0000418	109.9	0.0000
X_AX_B	0.0000187	1	0.0000187	49.2	0.0001
X_AX_C	0.0000397	1	0.0000397	104.5	0.0000
X_A^2	0.0000083	1	0.0000083	21.8	0.0016
X_B^2	0.0000111	1	0.0000111	29.2	0.0006
Blocks	0.0000078	2	0.0000039	10.2	0.0062
Lack of fit	0.0000190	19	0.0000004	2.6	0.0819
Pure error	0.0000030	8	13.4		
Total SS	0.000186	35			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

adjustability as described by the values in Table 4d.

In order to obtain an optimized model, desirability was calculated using the predicted model for stem crude juice, and the desirability index of the model was 72.59%. Derringer's desirability function was employed (Suich & Derringer, 1980). The function transforms the response of each variable into a desirability score, ranging between 0 (completely undesirable) and 5 (completely desirable), taking different forms depending on the used optimization criterion: maximization, minimization or attaining a fixed target. The best combination factors were established as desirable maximization turbidity in solution and minimization of relative enzymatic activity, total proteins and specific enzymatic activity since measured in the supernatant. The responses showed that, for the observed data, the more convenient result was that from experiment 3. However using the predict data from the adjusted model, the most suitable result was that from experiment 6 (coded responses, −1; 0; −1), as can be seen in Table 3. This prediction showed the values for optimum precipitation (coded responses, −0.233; −0.068; −1.0). When transporting the predict data to the un-coded values, the optimum values for BR isolation and recovery were 52.7 mg mL^{-1} of BR and 166.5 μg $mL^{-1}$of Carr at a pH of 4.5; In Table 3 it is possible to visualize the desirability of complex formation of each response. Fig. 4 shows all the surface models designs for all independent variables as well as its interactions when studying each dependent variable.

3.7. Peel crude juice

The statistical model applied to peels crude juice based on variance analysis was adjusted to the observed data. The variance of the samples was properly explained by the model since it presented an R^2 that varied between 70.86% and 86.53%.

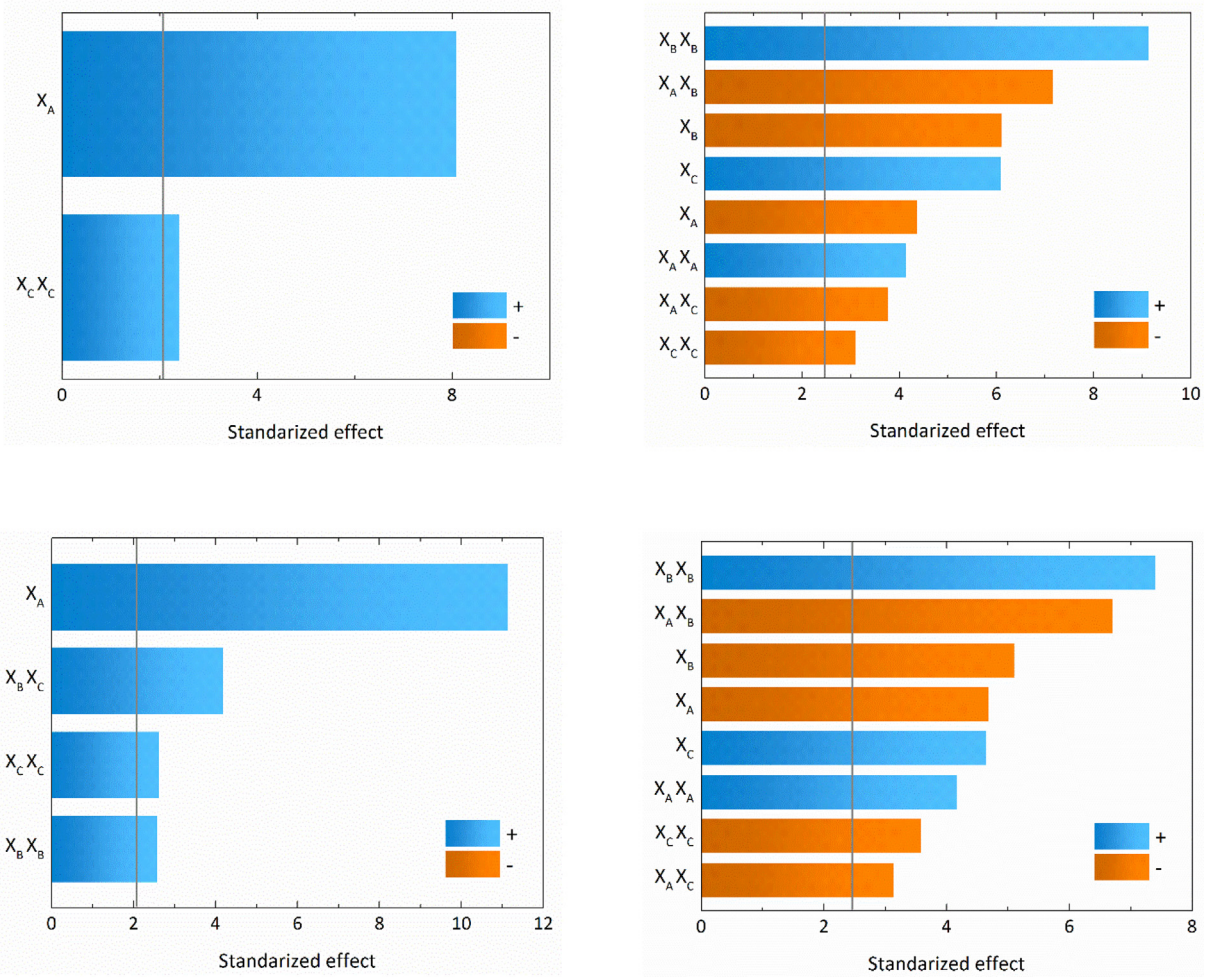

Fig. 5. Standardized Pareto charts encompassing the effect of each independent variable (i.e., (X_A) - BR (mg mL^{-1}), (X_B) - Carr (μg mL^{-1}) and (X_C) - pH, divided by its standard error, pertaining to four responses (i.e., turbidity, relative activity of BR (mg g^{-1}), total protein (mg g^{-1}) and specific activity of BR), obtained from pineapple peels crude juices. The vertical line in each chart represents the 10% significance level.

It can be seen that BR precipitation was affected mainly by the BR concentration (X_A), pH value (X_B). Carrageenan concentration (X_C) also affected but in a minor extension. The most important interaction parameters were X_AX_C, which means that the interaction between BR concentration and pH was the most important factor. The predicted response Y for the BR precipitation could be expressed by the following second-order polynomial equation in term of coded values:

$$Y(Turbidimetry) = 0.7942 + 0.1716X_A + 0.0750X_C$$
$$Y(Relative\ activity) = 12.78 - 6.47X_A - 9.03X_B + 8.75X_C - 15.40X_AX_B - 7.67X_AX_C + 8.89X_A^2 + 19.56X_B^2 - 6.67X_C^2$$
$$Y(Total\ protein) = 4308.21 + 745.40X_A + 397.625X_BX_C + 245.60X_B^2 + 258.56X_C^2$$
$$Y(Specific\ activity) = 0.003 - 0.002X_A - 0.002X_B + 0.002X_C - 0.004X_AX_B - 0.002X_AX_C + 0.003X_A^2 + 0.005X_B^2 - 0.002X_C^2 \quad (5)$$

As can be observed in Fig. 5, all the Pareto charts were organized in a decreasing order considering the impact of statistical significance. When studying turbidity, it was possible to verify that only 2 effects (X_A and X_C^2) influenced significantly the complex formation ($P < 0.05$), one linear and another quadratic coefficient, both with a positive effect. Through the analysis of the linear effect, it was possible to describe a proportional behavior towards turbidity formation, higher BR concentration in solution, higher the turbidity. The quadratic positive coefficient showed that the increase of pH did not affect linearly the turbidity since highest turbidity was achieved in the lower and higher ranges of pH tested (4.5 and 9.5), and the lower values of turbidity were achieved for pH 7. R^2 indicates that the adjusted model explains 70.86% of the variability of this dependent variable, and the adjustability value of 0.5386, explains that the model significantly fits the observed data (Table 6a).

The analysis of variance for enzymatic activity showed that only one effect (X_BX_C) was not significant, being removed from the model. Thus, 8 effects presented statistical significance ($P < 0.050$). Three of the nine coefficients presented a positive effect. The linear coefficient X_C showed a proportional increase of relative enzymatic activity with pH rise. Therefore, the lower pH allowed higher moving of enzyme from supernatant to precipitate. The quadratic coefficient X_A^2 and X_B^2 showed lower relative activity somewhere in between the tested ranges. Five factors presented a negative effect (X_A, X_B, X_AX_B, X_AX_C, and X_C^2), the two linear coefficients showed that the increase of each component decreased the amount of enzyme activity in supernatant in a quadratic manner, showing a non-linear behavior for enzymatic activity, which was expected since, higher concentration of polysaccharide in solutions leads to loss of enzymatic activity (Valetti et al., 2012). On the other hand, the formation of complex between protein and polysaccharide depends only on the columbic interactions, as previously described by Campos et al. (2017). Thus, when protein concentration increases, the ionic strength equilibrium changes, decreasing the complex formation and therefore a high concentration of protein is preserved free in solution.

The values of model fit are shown in Table 6b, which were sufficient

Table 6a
Analysis of variance (ANOVA) of Turbidimetry using crude juice from pineapple peel for the results of the BBD ($R^2 = 81.40$, $R^2_{adjusted} = 70.86$).

Factor	SS[a]	DF[b]	MS[c]	F	p
X_A	0.707	1	0.707	65.5	0.0000
X_C^2	0.063	1	0.063	5.6	0.0226
Blocks	0.255	2	0.128	11.8	0.0002
Lack of fit	0.130	13	0.010	0.93	0.5386
Pure error	0.291	27	0.011		
Total SS	1.446	44			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
$p < 0.05$ was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

Table 5
Range and codification criterion for independent variables in the BBD using crude juice from pineapple peel. Desirability values for an optimized model for isolation of BR.

Exp.	Coded values			Natural values			Turb	RelAct	TP	SpeAct
	X_A	X_B	X_C	X_A	X_B	X_C				
1	1	0	−1	80	125	4.5	1.10 ± 0.08	4.7 ± 3.1	6204	0.0009 ± 0.0007
2	0	−1	1	70	50	9.5	0.79 ± 0.16	44.0 ± 24.0	5725	0.0102 ± 0.0065
3	0	1	−1	70	200	4.5	0.86 ± 0.10	12.7 ± 1.2	4948	0.0030 ± 0.0006
4	0	0	0	70	125	7	0.89 ± 0.05	7.3 ± 1.2	5071	0.0017 ± 0.0002
5	1	0	1	80	125	9.5	1.11 ± 0.07	9.7 ± 3.8	6023	0.0019 ± 0.0009
6	−1	0	−1	60	125	4.5	0.77 ± 0.04	5.0 ± 1.0	4249	0.0013 ± 0.0002
7	−1	0	1	60	125	9.5	0.61 ± 0.07	41.0 ± 3.1	4731	0.0100 ± 0.0022
8	1	−1	0	80	50	7	0.80 ± 0.26	60.0 ± 25.5	5939	0.0153 ± 0.0079
9	−1	−1	0	60	50	7	0.63 ± 0.15	40.1 ± 30.1	4713	0.0109 ± 0.0082
10	0	−1	−1	70	50	4.5	0.90 ± 0.04	24.0 ± 8.1	5815	0.0050 ± 0.0024
11	0	1	1	70	200	9.5	0.82 ± 0.13	22.0 ± 2.0	6457	0.0043 ± 0.0011
12	1	1	0	80	200	7	1.01 ± 0.07	11.0 ± 2.7	6319	0.0020 ± 0.0006
13	0	0	0	70	125	7	0.90 ± 0.07	10.7 ± 1.2	4887	0.0024 ± 0.0003
14	−1	1	0	60	200	7	0.63 ± 0.10	52.0 ± 10.6	3885	0.0142 ± 0.0030
15	0	0	0	60	125	7	0.71 ± 0.13	20.3 ± 5.0	3953	0.0055 ± 0.0015

Variables	Factor values			Responses	Values
	Optimum	Low	High		Optimum
X_A (mg mL^{-1})	**78.5**	60.0	80.0	**Turb**	1.0165
X_B (μg mL^{-1})	**183.5**	50.0	200.0	**RelAct**	−0.5318
X_C	**4.5**	4.5	9.0	**TP**	5051
				SpeAct	−0.0009

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH. Turbidimetry (Turb), relative activity (RelAct), total protein (TP) and specific activity (SpeAct).

Table 6b
Analysis of variance (ANOVA) of Relative Activity using crude juice from pineapple peel for the results of the BBD (R^2 = 76.77, $R^2_{adjusted}$ = 76.27).

Factor	SS[a]	DF[b]	MS[c]	F	p
X_A	947.8	1	947.8	19.2	0.0047
X_B	1849.0	1	1849.0	37.4	0.0009
X_C	1837.5	1	1837.5	37.2	0.0009
X_AX_B	2544.9	1	2544.9	51.5	0.0004
X_AX_C	705.3	1	705.3	14.3	0.0092
X_A^2	852.9	1	852.9	17.3	0.0060
X_B^2	4125.8	1	4125.8	83.4	0.0001
X_C^2	479.9	1	479.9	9.7	0.0207
Blocks	85.4	2	277.2	5.6	0.0424
Lack of fit	364.4	27	139.3	2.82	0.0993
Pure error	120.3	6	49.4		
Total SS	3168.9	43			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

Table 6d
Analysis of variance (ANOVA) of Specific Activity using crude juice from pineapple peel for the results of the BBD (R^2 = 76.99, $R^2_{adjusted}$ = 76.16).

Factor	SS[a]	DF[b]	MS[c]	F	p
X_A	0.000100	1	0.000100	22.0	0.0034
X_B	0.000119	1	0.000119	26.2	0.0022
X_C	0.000098	1	0.000098	21.7	0.0035
X_AX_B	0.000205	1	0.000205	45.0	0.0005
X_AX_C	0.000045	1	0.000045	9.9	0.0201
X_A^2	0.000079	1	0.000079	17.4	0.0059
X_B^2	0.000250	1	0.000250	54.9	0.0003
X_C^2	0.000059	1	0.000059	12.9	0.0115
Blocks	0.000101	2	0.000011	11.2	0.0095
Lack of fit	0.000019	28	0.0000004	2.4	0.1374
Pure error	0.000003	6	13.4		
Total SS	0.001404	44			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

to indicate that the model is adequate to the observed data. Consequently, studying the best features for this response (minimizing the relative enzymatic activity on supernatant) the exact predicted values were 0.20; 0.31; −1.00 (coded responses), the un-coded responses represented 57 mg mL^{-1} of BR and 137,5 μg mL^{-1} of Carr at pH 4.5.

The analysis of variance of the response – total protein showed that 4 effects (X_A, X_BX_C, X_B^2 and X_C^2) were statistically significant ($P < 0.050$) and the values are described in Table 6c. All the significant factors presented a positive effect towards the response. The increasing of protein in solution represented an increase of free total protein in the supernatant after precipitation process, which represents a linear behavior. The interaction between the increasing of Carr in solution and pH value was very important since presented an inversely proportional behavior, but not linear, as can be proved by the quadratic coefficients of the two factors. The best feature (lower amount of total protein on the supernatant) was achieved when the pH value was near the minimum range and the Carr concentration was near 125 μg mL^{-1}.

The adjusted model indicated that the observed data was adequate at a 5% significance level and the variance of the response was explaining for the observed data.

The specific enzymatic activity evaluated the amount of remaining active enzyme at the supernatant, taking into account the total protein in the solution, and the statistical analysis of the model showed that eight of the nine effects were statistically significant ($P < 0.050$). Thus, the not significant effect (X_BX_C) was removed from the final statistical model. The values of model fit are described in Table 6d. After analysis X_C, X_A^2 and X_B^2 presented positive effects. The linear effect of pH showed an increase of specific enzymatic activity in the supernatant with an increase of the factor, but the analysis of the quadratic coefficient showed a negative effect for higher pH, which means that the amount of BR in solution reaches a plateau, followed by a decrease. In contrast, the other two quadratic effects (BR and Carr concentration) presented a positive effect, but for linear coefficients, these showed a negative effect. The increase of BR and Carr concentrations led to a decrease of BR activity in the supernatant, which indicates that higher amounts of complex were produced and precipitated, until a minimum plateau in the BR activity was reached. These results are in accordance with those described by Campos et al. (2017), where the authors described the mechanisms of complex formation between BR-Carr. Thus, with these results, it is possible to demonstrate that when the minimal plateau is reached for polysaccharide concentration, the BR stopped to complex with Carr and started to move again to the soluble complex and then releases from the complex.

Table 6c
Analysis of variance (ANOVA) of Total Protein using crude juice from pineapple peel for the results of the BBD (R^2 = 88.49, $R^2_{adjusted}$ = 86.53).

Factor	SS[a]	DF[b]	MS[c]	F	p
X_A	0.00000013	1	0.00000013	124.2	0.0000
X_BX_C	0.0000019	1	0.0000019	17.7	0.0003
X_B^2	722289.0	1	722289.0	6.7	0.0159
X_C^2	744923.0	1	744923.0	6.9	0.0145
Blocks	0.00000011	2	0.0000056	52.1	0.0000
Lack of fit	397090.0	14	124888.0	0.91	0.3603
Pure error	0.0000026	24	107363.0		
Total SS	0.0000003	44			

Abbreviations: (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH.
p <0.05 was considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean squares.

The optimum characteristics of the model were determined by a desirability index of 80.00%, and as observed in Fig. 3, it was possible to visualize the desirability of complex formation of each factor. The same desirability function was employed to calculate the best optimized model, and desirable values to achieve the maximization of turbidity in solution and minimization of relative enzymatic activity were established based on total proteins and specific enzymatic activity in the supernatant. Using the predicted data from the adjusted model, the best results were those obtained from experiment 16 (coded responses, 1; 0; −1), but when calculating the exact predicted values for the best conditions to move BR from supernatant to a precipitate the best results, were 0.858; 0.778; −1.0 (coded responses), with the un-coded responses represented by 78.5 mg mL^{-1} of BR and 183.5 μg mL^{-1} of Carr, at pH 4.5. Fig. 6 shows all the surface models designs of each independent variable as well as their interactions when studying each dependent variable. The values of coefficients are shown in Table 5.

Taking into account the differences between crude juices, it was possible to conclude that higher amount of Carr is needed to precipitate BR in peel crude juice, which showed the lower amount of BR. This probably occurs due to the presence of other components that may interact with complex formation. These results show that the biological precipitation method is stable using slightly different crude juices, with the potential to be widely applied in the separation, concentration, and recovery of other natural products with similar chemical properties.

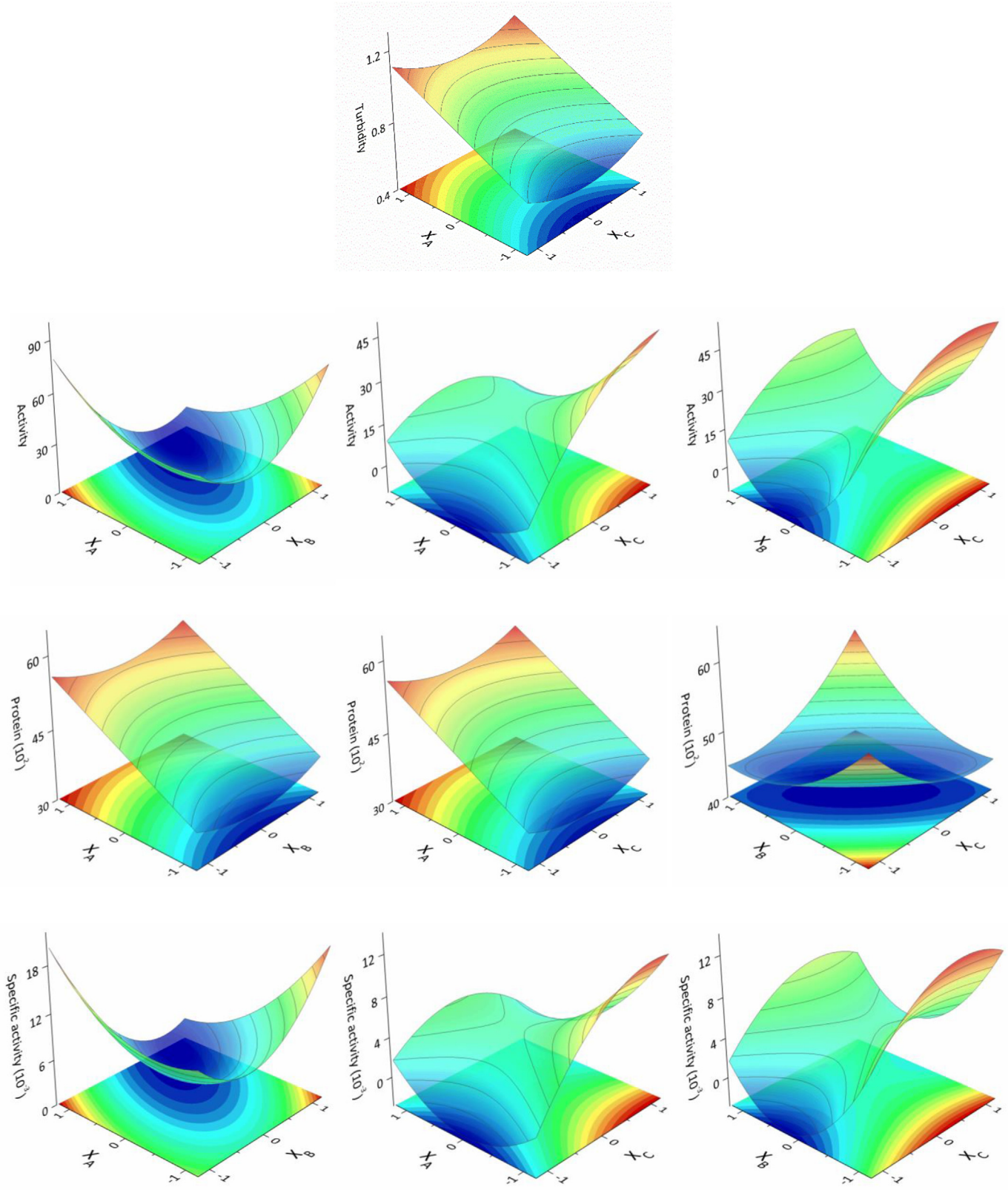

Fig. 6. Response surfaces corresponding to the combined effect of (X_A) - BR concentration (mg mL^{-1}), (X_B) - Carr concentration (μg mL^{-1}) and (X_C) - pH. Turbidimetry (Turb), on the maximum passage of BR towards the precipitate through the formation of complex with Carr, according to equation (5). Four responses were evaluated (i.e., turbidity, relative activity of BR (mg g^{-1}), total protein (mg g^{-1}) and specific activity of BR), obtained from pineapple peels crude juices.

3.8. Proteolytic activity of pineapple byproducts crude juices

After model application and optimization of complex formation between enzyme and polysaccharide, it was possible to measure the proteolytic activity of each juice. Thus, at pH 9 and in order to make a comparison with standard enzymatic formulations, the peel crude juice presented a 3.1 U mg^{-1} protein and the stem crude juice presented 2.7 U mg^{-1} of protein. These values are in accordance with those described for standard bromelain (Sigma–Aldrich (St. Louis, Missouri, USA)), which present ≥3 U mg^{-1} of protein. Nevertheless, in this case, it should be highlighted a low-cost technology was applied, without the use of organic solvents or inorganic salts and obtaining the same degree of proteolytic activity, but higher purity degree. Mohan, Sivakumar, Rangasamy, and Muralidharan (2016) described the proteolytic activity of peels and stems crude juices from pineapple, with respective values of 4.7 U mL^{-1} and 4.5 U mL^{-1} of crude juice, respectively, at pH 4.5. In comparison with those obtained through Carr precipitation for the same pH range, the peel presented 9.3 U mL^{-1} and for stem it presented 11.6 U mL^{-1} of crude juice, which clearly showed the increase of proteolytic activity per mL of crude juice.

4. Conclusion

The use of natural crude juices to recover proteins by electrolyte precipitation allows decreasing the recovery costs as well as the environmental impact while achieving good extraction yields and high enzyme purity. The previously tested biological precipitation system with Carr was now applied to stem and peel pineapple crude juices, in order to purify BR, an important enzyme. To this research was demonstrated that Carr allows to efficiently precipitate BR from crude juices. Through polysaccharide precipitation, it is possible to maintain the biological activity of BR. Besides that, the technology presents a high yield of extraction (ca. 0.3 g of BR/100 g of pineapple byproducts) and the enzyme activity recovery yield is between 80 and 90%.

A pH value of 4.5 is the best for the extraction of BR from both stems and peels. On the other hand, the best conditions for enzyme precipitation from stems is 52.7 mg mL^{-1} of BR for 166.5 μg mL^{-1} of polysaccharide, with 2.7 U mg^{-1} protein of proteolytic activity, and for peel crude juice the best precipitation occurs with 78.5 mg mL^{-1} of BR in solution and 183.5 μg mL^{-1} of Carr, with 3.1 U mg^{-1} protein of proteolytic activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodhyd.2018.09.009.

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