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ORIGINAL PAPER

Characterising polysaccharides in cherimoya (*Annona cherimola* Mill.) purée and their enzymatic liquefaction

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Abstract Polysaccharides isolated in the alcohol-insoluble residues (AIR) from cherimoya (*Annona cherimola* Mill.) were characterised for contents of soluble pectin, cellulose, hemicellulose and lignin, and the distribution of neutral sugars and uronides in water-soluble pectin (WSP) and water-insoluble AIR (WAIR) fractions. For WSP, the predominant neutral sugar was arabinose and, for WAIR, cellulosic glucose and xylose. Two enzyme preparations were tested for their capacity to release neutral sugars and uronides from WAIR. The optimal incubation temperature (45 °C) and the most effective preparation—rich in pectinase, cellulase and xylanase activities—were selected according to a central composite rotatable design (CCRD). Enzyme was also applied to native cherimoya purée according to another CCRD, varying the enzyme concentration and incubation time. Native purée exhibited strong shear-thinning behaviour with high, extrapolated, yield stress. During enzymatic treatment, behaviour was less shear thinning, and yield stress, consistency index and Bostwick consistency tended to decrease, giving rise to purées of different rheological properties.

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Introduction

Cherimoya (*Annona cherimola* Mill.) originates in the Andes, probably in a region straddling southern Ecuador and northern Peru [21], where it is found growing at altitudes between 1,300 and 2,200 m above sea level (masl). The fruit is commercially significant in Andean countries, particularly Ecuador, Peru and Chile. It is also commercially cultivated in subtropical Australia, Spain, Italy and California (USA) [15]. Production in Ecuador alone is estimated as being about two thousand tons and a cultivated surface around1,500 ha. The subtle aroma, flavour and whiteness of the pulp provide the main interest of the fruit, giving it high commercial potential, especially for niche export markets [26]. Cherimoya is a much smaller fruit than its relative, soursop (*A. muricata* L.), and it is regarded as having slightly more flavour.

Despite being climacteric [13], the cherimoya is difficult to handle and transport because it is highly sensitive to mechanical damage [28]. The main obstacles to the fruit's successful marketing are perishability and susceptibility to mechanical and physiological damage (chilling injury) [1, 15]. The fruit is also very sensitive to external damage caused by fruit flies and anthracnose. Consequently, large amounts of fruit are discarded at the plantation because of problems with external appearance. A valuable alternative is to process fruits that do not qualify for the fresh market. Interest is therefore high in cherimoya purée as a raw

material for juice and for mixing with dairy products to develop flavoursome and healthy food items.

However, several constraints exist to the industrial processing of cherimoya. One is the very rapid enzymatic browning of the pulp, which becomes pink when exposed to oxidation on extracting and separating the arils [18]. The second constraint is the very high viscosity of the purée, whether obtained mechanically or manually, which further limits processing and use by fruit industries.

Rheological properties of fruit products are attributed mainly to the polysaccharides that constitutes the cell walls in the fruit [27]. The characterisation of polysaccharides in the cell walls of cherimoya fruit has not yet been published, even though it would help better define the appropriate enzymatic cocktail for obtaining purées with different rheological properties and, consequently, expanding the use of cherimoya purées in food products.

Materials and methods

Raw materials

Cherimoya fruits were harvested in a region of the Department of Pichincha at 1,300–2,200 masl in the Central Cordillera of the Ecuadorian Andes. Only fully mature fruit with soluble solids between 19 and 23 g 100 g^{-1} and with no apparent injuries were harvested at random from a commercial plantation that follows INIAP's crop management recommendations. On the day of harvest, the fruits were peeled by hand, and their seeds and central receptacle removed manually. The fruit was then puréed, homogenised and kept frozen in small bags at –25 °C.

Preparing cell-wall materials

Alcohol-insoluble residues (AIR), water-soluble pectin (WSP) and water-insoluble AIR (WAIR) were recovered according to previously described procedures [25]. Briefly, cherimoya pulp was suspended in five volumes of hot 95% ethanol. The slurry obtained was filtrated and solid residue washed thoroughly with an alcoholic solution (80/20% ethanol/water) and freeze-dried. Then it was incubated with a protease (pronase®) derived from *Streptomyces griseus* (Boehringer-Mannheim, Germany). It was then mixed with a thermostable α-amylase (Thermamyl Liquid 60® from Novo Industrials, Denmark) and an amyloglucosidase from *Aspergillus niger* (Merck, Germany) according to Brillouet et al. [5]. The slurry was subsequently deproteinated and destarched, centrifuged and washed thoroughly with alcohol to obtain AIR. The hydrosoluble part of AIR (WSP) was obtained by washing AIR thoroughly with cold distilled water. The residues (WAIR), corresponding to insoluble cell-wall polysaccharides, were freeze-dried, milled and sieved (mesh size <500 μm).

Physico-chemical analysis

Cherimoya purée was analysed for pH, titratable acidity, dry matter, and ash using standard methods [2]. Total soluble solids were assessed with a hand refractometer (Atago, Japan) and result expressed in g 100 g^{-1}. Sugars (glucose, fructose and sucrose) were determined following the method of [10] using a HPLC featured with a column BIORAD-AMINEX HPX 87.Vitamin C was assessed by the reflectometric method, using an ascorbic acid test (Merck Reflectoquant® kit). Cellulose and hemicellulose were extracted by selective fractionation of WAIR according to methods described by Voragen et al. [27]. Lignin was determined according to Effland [9]. Proteins were estimated, using the Kjeldahl method, and residual starch was assessed by the method described by Batey [3]. Neutral sugars were analysed by gas chromatography (GC) after hydrolysing residues by either the Saeman procedure with concentrated sulphuric acid (72%) or the trifluoroacetic acid (TFA; 2 mol L^{-1}) method, followed by conversion of sugars to alditol acetates and subsequent analysis by GC (Shimadzu GC-14B, with an FID detector and featuring a Supelco SPB-225 capillary column [930 m × 0.32 mm]) according to methods adapted from Brillouet et al. and Harris et al. [5, 11]. Only the higher contents of neutral sugars obtained were reported, whether by Saeman or TFA hydrolysis. Glucose liberated by TFA hydrolysis was reported as non-cellulosic glucose (NC glu), whereas cellulosic glucose was estimated as the difference between the TFA and Saeman hydrolyses.

Total neutral sugars were measured, using the anthrone method [8] and deducing from interference of galacturonic acid. Uronides were determined by the *m*-hydroxy-diphenyl spectrophotometric method [4]. Degree of esterification of galacturonic acid was performed, as described by Klavons and Bennet [12]. Suspended insoluble solids (SIS) were assessed as the percentage weight of the residue after centrifuging cherimoya purée at 1,000*g* for 15 min.

Viscosity was assessed with a controlled shear rate Brookfield viscometer (model DV-II+, Brookfield Engineering Laboratories, Inc., MA, USA), featuring a thermostatic bath. Shear stress (σ in Pa) was measured

at different wall shear rates (γ), ranging from 0.21 to 85 s^{-1}. The experimental data were fitted to a three-parameter Herschel–Bulkley model (Eq. 1), with the help of the software SigmaPlot (v.7.1; SPSS, Inc., IL, USA) and applying a non-linear regression model through the Levenberg–Marquardt algorithm for determining yield stress (σ_0), consistency index (K) and flow behaviour index (n):

$$\sigma = \sigma_0 + K\gamma^n \quad (1)$$

Consistency was assessed with a Bostwick consistometer, using 90 g of purée at ambient temperature (20 °C), and measuring the distance (in cm) that the pulp flowed under its own weight on a 15° slope for 30 s [22].

Enzymatic activity

Activity of pectin lyase (PL; EC 4.2.2.10) was measured at 40 °C in a highly esterified (80%) citrus pectin solution dissolved in a 0.5% citrate phosphate buffer (pH 6.0) [7]. Endo-Polygalacturonase (PG; EC 3.2.1.15) was assayed, using polygalacturonic acid as a substrate in acetate buffer (pH 4.2, 30 °C). Reducing groups were determined by the Nelson method, as modified by Liu and Luh [14]. Pectinesterase (PE; EC 3.1.1.11) activity was determined by titrating the carboxylic groups released, continually adding 0.1 M NaOH at a constant pH 6.0 and 30 °C, and using pectin solution as substrate [16].

Activity of endo-1-4-β-D-glucanase (C_x; EC 3.2.1.4) was measured in acetate buffer (pH 4.6, 38 °C), using medium viscosity carboxymethylcellulose sodium salt (Sigma C-4888) as a substrate and activity of the 1,4-β-D-glucan cellobiohydrolase (C_1; EC 3.2.1.91) was measured in the same buffer (pH 4.8, 50 °C), using insoluble crystalline cellulose as a substrate, both method according to [17]. For both determinations, reducing sugars were measured by the dinitrosalicylic acid (DNS) method [19].

The enzymes (1,4)β-D-xylanase (EC 3.2.1.8); (1,4)β-D-mannanase (EC 3.2.1.78); and (1,4)β-D-galactanase (EC 3.2.1.89) were assessed, using Azurine-cross-linked (AZCL) to purified polysaccharides as insoluble chromogenic substrates, that is, respectively, AZCL-xylan, AZCL-galactomannan and AZCL-galactan (all from Megazyme International Ireland, Ltd.), at 2% (w/v) in acetate buffer (25 mM) at pH 4.7, 4.5 and 4.3, respectively. Substrate solutions with the enzymatic cocktail were incubated at 40 °C for 10 min. The high molecular substrates were then removed, employing 2.5 volumes of ethanol at 95% (v/v) and centrifuging at 1,000g. In the supernatant, the absorbance at 590 nm of the released dye-labelled polysaccharide fragments was measured. Enzymatic activity was determined by reference to a standard curve provided by the manufacturer (Megazyme International Ireland, Ltd.). Activity α-L-arabinofuranosidase (EC 3.2.1.55) was assessed, using as substrate p-nitrophenyl-α-L-arabinofuranoside (Sigma N-3641) at 0.1% in acetate buffer (0.1 M at pH 4.5 and 40 °C) and reading at 400 nm the p-nitrophenol released [23].

Enzymatic treatment

The enzyme preparations known by their commercial name, Rapidase Carrot Juice® and Rapidase Pomaliq 2F® were purchased from DSM (Seclin, France).

Enzymatic hydrolysis of WAIR was performed as follows: 10 mg of WAIR was suspended in 10 mL of phosphate buffer at the fruit's pH (pH 4.6). An enzyme preparation was added and the final concentration was reported as equivalent to μL kg^{-1} of cherimoya purée, taking into account its percentage of WAIR. During incubation, temperature was controlled and constant magnetic stirring was applied. Samples were then heated for 5 min at 80 °C to inactivate the enzymes, and the slurry centrifuged at 6,000g for 10 min. Supernatant was recovered for subsequent analysis of the released sugars. The enzyme preparations used had been previously assessed for their endogenous contents of AGU and neutral sugars. Thus, only sugars released from the WAIR were reported.

Assays on native cherimoya purées were done by introducing 1 mL of diluted enzyme solution to 100 g of purée, incubating at 45 °C with gentle magnetic agitation. The purées were then pasteurised at 80 °C for 5 min for later analysis.

Experimental designs

Two central composite rotatable designs (CCRD) were implemented, both with two variables (X_1 and X_2). The variables for the first CCRD implemented, corresponded to temperature (X_1) and enzyme concentration (X_2) for assessing the response pattern of WAIR solubilisation. The variables of the second one, corresponded to time (X_1) and enzyme concentration (X_2) for estimating responses during the liquefaction of native cherimoya purée. Responses (Y) were computed, using statistical software (JMP® 5.1; SAS Institute, Inc., NC, USA) to fit a second-order polynomial equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2. \quad (2)$$

Statistical tests (R^2), probability (P) that one factor of the model is different from zero, and probability that of lack of fit (P_{lof}) is zero were calculated by the same software. Response surfaces were presented as contour plot graphs, using SigmaPlot (v.7.1; SPSS, Inc., IL, USA).

Results and discussion

Physico-chemical characterisation of cherimoya fruits

The edible part of cherimoya (var. Lissa)-juicy white arils-represents about 72.6 ± 1% of the entire fruit, and has high value for industrial purposes. The thin greenish rind, the oblong black seeds embedded in the arils and the fibrous central receptacle of fused carpels, represent, respectively, only 20.4, 6.5 and 0.6 ± 1% of the total weight of the fruit.

Table 1 presents the chemical composition of the edible part of the fruit (mesocarpe). It shows that the cherimoya variety being evaluated in this study is sweeter than other previously studied varieties [20], as total soluble solids amount to 21 g 100 g^{-1}. Analyses also show that cherimoya is a good source of vitamin C and minerals. Residual starch is also present in mature fruits (0.4%), although in lower quantities than in soursop, a fruit that belongs to the same Annonaceae family [25]. Cherimoya fruit also has a high pH, which means that the juice must be slightly acidified for better preservation.

Characterising cell-wall polysaccharides

The fleshy arils in which the seeds are embedded are fibrous and juicy. The AIR obtained after the partial removal of starch and cytoplasmic protein represents about 4.3 ± 0.3 g 100 g^{-1} of the fruit's edible parts (fresh basis). This is a relatively high level, compared with other fruits [27], although it is much the same as for soursop [25]. The edible part of cherimoya can be considered as a good source of fibre and, especially, soluble pectin (WSP) and cellulose, which yield, respectively, 29 and 20.9% of total AIR (Table 2). Lignin content is also important, representing almost 15.9% of purified cell walls. Indeed, cherimoya is claimed to be rich in sclereids, which are highly lignified cells that are also common in pears [24]. Hemicellulose and insoluble uronides, respectively, represent only 12.2 and 3.5% of the cell walls. Actually, the water insoluble part of AIR, which corresponds to WAIR, yields 71% of total amount of AIR (Table 2).

WSP is composed mainly of uronides (53% of mole fraction), 84% of which are esterified, and arabinose, the predominant neutral sugar in the pectin side chain (19.3% of mol) and representing almost 41.1% of all neutral sugars in WSP (Table 3). It is followed by glucose (12.5% mol) and galactose (6.5% mol). All other sugars represent less than 10% mol of total sugars. The predominance of arabinose in the pectin side chain has been also noted previously in soursop [25] and may be a peculiarity of fruits of the Annonaceae family.

In the WAIR, glucose represents the major sugar, which agrees with the high cellulose content. The predominant sugar after glucose is xylose, which represents half of the non-cellulosic sugars and proves that polysaccharides with high xylose content (probably xylane) also constitute cell walls in cherimoya. The presence of insoluble pectic polysaccharides is low in WAIR, as indicated by the uronide content (5.3 g 100 g^{-1}) with respect to total amount of neutral sugars (48 g 100 g^{-1}).

Table 1 Main physico-chemical characterisation of cherimoya purée (fresh basis; standard error, $n = 3$)

Analysis	Units	Cherimoya purée
Total soluble solids	g 100 g^{-1}	21.06 ± 1.95
pH		4.64 ± 0.01
Firmness	kg cm^{-2}	1.25 ± 0.40
Titratable acidity	g 100 g^{-1} equ. citric acid	0.33 ± 0.01
Dry matter	g 100 g^{-1}	22.49 ± 1.04
Ash	g 100 g^{-1}	0.87 ± 0.06
Vitamin C	mg 100 g^{-1}	61.48 ± 2.29
Starch	g 100 g^{-1}	0.44 ± 0.06
Sucrose	g 100 g^{-1}	6.50 ± 0.5
Glucose	g 100 g^{-1}	4.04 ± 0.4
Fructose	g 100 g^{-1}	4.45 ± 0.4

Standard deviation calculated with 3 repeats on 3 blocks of an average 30 fruits

Enzymatic liquefaction of isolated cell-wall polysaccharides

Various commercial enzyme preparations were tested at different concentrations and at different temperatures for their capacity to solubilise insoluble cell walls (i.e. WAIR). The percentage of uronides and neutral sugars released was also assessed. Enzyme preparations with only pectinase activities failed to release neutral sugars and uronides in any significant amount (data not shown). Only the enzyme preparations containing both high pectinolytic and celulolytic activities (Table 4) worked, as shown in Fig. 1.

In Fig. 1, is represented the contour plots of the surface responses generated with good fitting values

Table 2 Main component of alcohol-insoluble-residue (AIR) of cherimoya purée (mesocarp; SE, $n = 3$)

In AIR (g 100 g^{-1} in dry AIR)									Total
WSP	WAIR								
	Uronides[a]	Cellulose	Hemicellulose	Lignin	Protein	Residual starch	Ash	Tannins	
29 ± 1	3.5 ± 0.1	20.9 ± 0.1	12.2 ± 0.1	15.9 ± 0.2	4.6 ± 0.1	4.5 ± 0.01	0.5 ± 0.01	0.005	91.1

[a] Including methanol

Table 3 Sugar composition of water-soluble pectin (WSP) and purified insoluble cell walls (WAIR) of cherimoya mesocarpe

Chirimoya	Rha[a]	Fuc[a]	Ara[a]	Xyl[a]	Man[a]	Gal[a]	Glu[b]	Glu.N[c]	Uronides
%mol in WSP (g 100 g^{-1} WSP)									
Mesocarpe	1.88 (1.04)	0.45 (0.25)	19.29 (9.75)	4.75 (2.4)	1.52 (0.92)	6.57 (4.0)	12.45 (7.55)		53.09 (DE 84%) (36.5)
%mol in WAIR (g 100 g^{-1} WAIR)									
Mesocarpe	0.69 (0.35)	0.94 (0.48)	4.58 (2.13)	25.35 (11.78)	3.48 (1.94)	2.87 (1.6)	45.36 (25.3)	7.96 (4.44)	8.77 (5.3)

DE degree of esterification

[a] Only highest values obtained from both Saeman and TFA hydrolyse was reported

[b] Cellulosic glucose (Glu) was obtained by differences between hydrolyse with H_2SO_4 and TFA

[c] Non-cellulosic glucose was given by hydrolysis with TFA

Table 4 Characterisation of activities (nkat mL^{-1}) of two of the commercial preparation used

Activity	Rap. carrot[a]	Rap. pomaliq[b]
Pectinases		
Pectin lyase	834 ± 100	1,100 ± 150
Polygalacturonase	29,100 ± 3,000	8,770 ± 1,000
Pectin-methyl esterase	4,330 ± 500	750 ± 100
Cellulases		
Endoglucanase	3,420 ± 300	3,300 ± 400
Cellobiohydrolase	5,120 ± 500	6,370 ± 700
Segondary activities		
Xylanase	1,384 ± 150	6,501 ± 600
Mannanase	500 ± 50	800 ± 100
Galactanase	730 ± 100	780 ± 100
Exo-arabinase	550 ± 50	100 ± 10

($R^2 > 0.95$; $P < 0.01$; $P_{lof} > 0.4$) from the experimental designs realised. It can be observed that after 90 min of incubation with Rapidase® Pomaliq 2F, the levels of neutral sugars and uronides released represented as much as 20 and 4.5% of total weight of WAIR, respectively, given an optimal incubation temperature of 45 °C and an enzyme concentration that is equivalent to 500 µL kg^{-1} of cherimoya purée. The same optimal temperature for liquefaction of other fruits, using commercial pectinolytic enzymes, had also been found previously [6]. According to the WAIR's chemical composition (Table 3), almost 41 and 85% of the total weight of respectively, neutral sugars and uronides in WAIR, can be released with the help of these enzymes at the highest concentration range. For both uronides and neutral sugars, both enzymes worked similarly but with a significant difference in favour of Rapidase Pomaliq 2F®. Indeed, cellulase and pectinase activities appear to be very similar for Rapidase Pomaliq 2F® and RapidaseCarrot Juice® (Table 4), except the latter exhibited higher levels of polygalacturonase and pectinesterase. This difference, however, does not appear to be an advantage for liquefying cherimoya WAIR. A higher content of xylanase activity for Rapidase Pomaliq 2F® may explain the difference observed, if we consider that polysaccharides with high xylose content are predominant in cherimoya cell walls (Table 3).

Applying enzymes to cherimoya purées

Rapidase Pomaliq 2F® was then chosen for the liquefaction of native cherimoya purée at the optimal temperature (45 °C). Some rheological properties were studied on treated purée according to enzyme concentration and incubation time. Table 5 shows that native cherimoya purée without enzymatic treatment (experiments 1 and 4) is very consistent, as it does not even flow on the Bostwick consistometer. The untreated cherimoya purée contains about 94% of SIS, which is extremely high. When modelling shear stress/shear rate, using the Herschel–Bulkley model (Eq. 1), the native purée exhibits a high shear-thinning behaviour (flow index $n = 0.27 \pm 1$), a high consistency index (27–30 Pa s^{-n}) and a relatively high extrapolated yield stress ($38 < \sigma_0 < 43$ Pa).

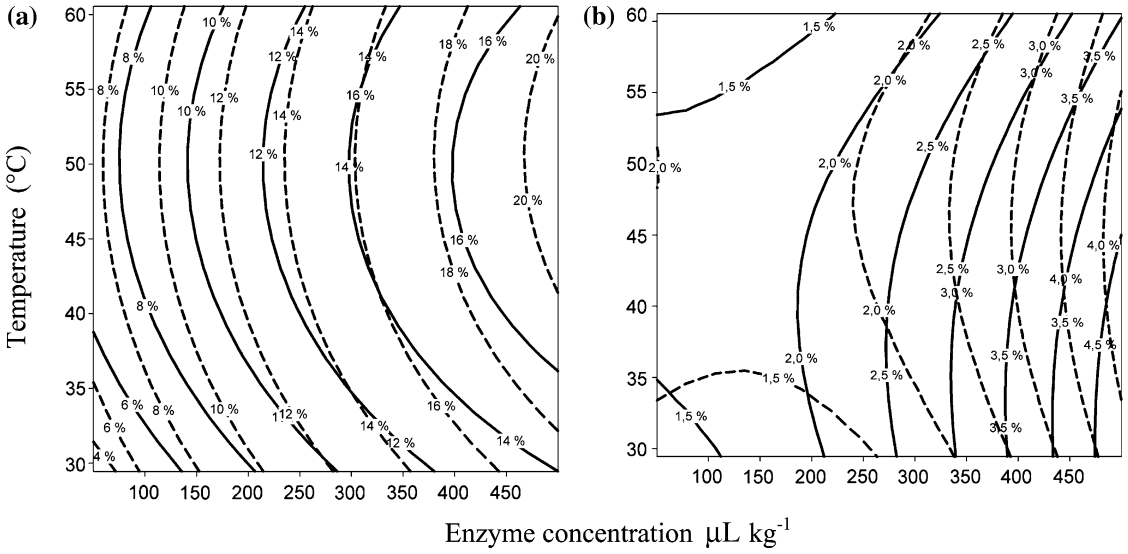

Fig. 1 Solubilisation of neutral sugars (**a**) and galacturonic acid (**b**) (as % weight of WAIR) obtained after 90 min of incubation at pH 4.6 with Rapidase carrot® (*solid line*) and Rapidase Pomaliq® (*dashed line*)

The Herschel–Bulkley model appears to fit well for all treated and untreated purées in the range of the shear rates studied ($R^2 > 0.98$, $P < 0.01$; Table 5). A higher dynamic yield stress is recorded for untreated juice, thus agreeing with the observation made with the Bostwick consistometer, as the shear stress value induced by gravity was probably below the yield stress. The cherimoya purée was therefore unable to flow and behaved, instead, like an elastic solid.

Following the response surface method, the Bostwick consistency index, percentage of SIS, yield stress (σ_0), consistency coefficient (K) and behaviour index (n) were modelled as a function of incubation time and activity, following a second-order polynomial equation (Eq. 2). Fitting was considered as acceptable for all responses ($R^2 > 0.88$; $P < 0.06$; $P_{lof} > 0.16$). The contour plot of the modelled response surface is shown in Fig. 2. Figure 2a shows that the flow velocity on the Bostwick consistometer increases almost in the same way with either incubation time or enzyme concentration. The same pattern is followed by SIS, which decreases when both factors increase, demonstrating good correlation between solid fraction content and consistency.

In Fig. 2b, the contour plot shows that, for purées undergoing strong enzymatic treatment, yield stress (σ_0) and the consistency coefficient K tended to decrease to a minimum, that is, to 5 Pa and 10 Pa s^{-n}, respectively. Likewise, the more heavily treated purée tends to exhibit less shear-thinning behaviour as behaviour index n increases.

Conclusion

Native cherimoya purée is characterised by very high contents of cell-wall polysaccharides, with xylose, the predominant non-glucosic sugar in the insoluble cell walls. Even so, through appropriate enzymatic treatment, purées of different rheological properties can be obtained that would significantly extend the uses of this

Table 5 Responses applying the CCRD for enzymatic liquefaction of cherimoya purée varying enzyme concentration and incubation time (temperature 45 °C)

Experiment	Concentration (µL kg^{-1})	Time (min)	Consistencia cm min^{-1}	Solubilised SIS (%)	Herschel–Bulkley model (Eq. 1)			
					σ_0	K	n	R^2
1	0	35	0	0	43	27	0.27	0.99
2	50	10	0.5	10.2	39	28	0.275	0.98
3	50	60	0.5	6.5	10.5	15.7	0.36	0.98
4	175	0	0	2	38.2	30	0.28	0.99
5	175	35	1.2	11.7	8	20.4	0.36	0.99
6	175	35	1	10.5	11.1	16.4	0.35	0.99
7	175	70	1.5	16.8	5.7	9	0.37	0.99
8	300	10	0.5	7.8	19	16	0.36	0.99
9	300	60	2	23	6.8	13.5	0.33	0.99
10	350	35	1.5	15.2	5.7	9	0.37	0.99

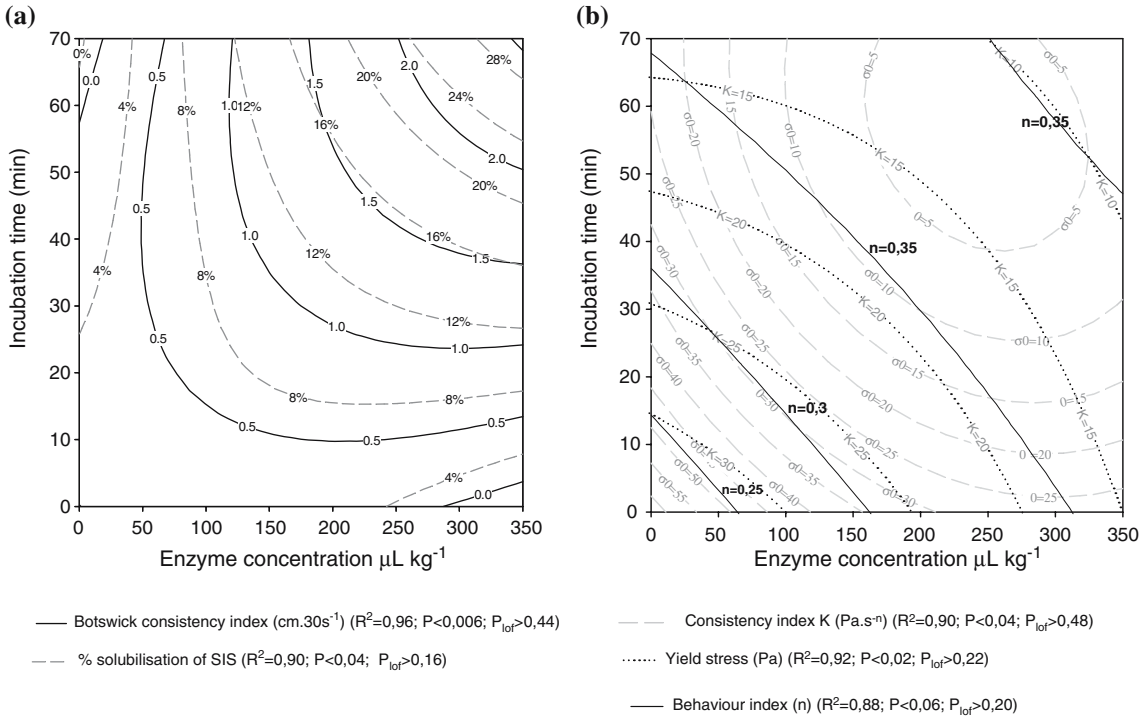

Fig. 2 Contour plot of Bostwick consistency index, % solubilised SIS, yield stress (σ_0), consistency index (K) and flow behaviour index (n)

fruit juice in food products and make its processing easier. Enzymatic treatment with a commercial preparation that contains high levels of pectinase, cellulase and xylanase activities appears to be effective, both on isolated insoluble cell walls and consequently on native cherimoya purée.

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