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Extraction and Characterization of Bromelain from Pineapple Core: A Strategy for Pineapple Waste Valorization

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Abstract: Bromelain is a mixture of cysteine endopeptidase usually extracted from pineapple juice and is used for the treatment of various human diseases and in industrial applications. Bromelain demand is quickly increasing, and its recovery from pineapple residues appears to be a sustainable waste management strategy. Pineapple core is among the most significant waste materials in the production of canned pineapple and is richer in bromelain than other pineapple residues. In this project, we compared the enzymatic properties and composition of bromelain extracts from either pineapple core or pulp to address the recovery of bioactive bromelain from pineapple core, thus contributing to the valorization of this waste material. Although significant differences were detected in the protein content of the two preparations, no differences could be detected for their proteolytic activity and for the effect of pH on their enzymatic activity. Mass spectrometry (MS) approaches identified the same peptidases in the fruit and in the core. This confirmed the possibility of using pineapple core to obtain relevant amounts of bioactive bromelain by applying a relatively simple procedure, thus paving the way to implementing a circular economy in this specific industrial sector.

Keywords: bromelain; pineapple core; proteolytic activity; mass spectrometry; waste valorization

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1. Introduction

Bromelain is a well-known mixture of different cysteine endopeptidases from pineapple and has been widely used for the treatment of various human diseases due to its demonstrated anti-inflammatory, anti-thrombotic, anti-edematous and fibrinolytic activities that have made pineapple quite popular in traditional medicine [1,2]. Bromelain anti-inflammatory and analgesic activity was first reported for the treatment of both osteoarthritis and rheumatoid arthritis [3] and was confirmed by several studies afterward [4,5]. Bromelain was effective in the treatment of acute sinusitis, rhinitis, and chronic rhinosinusitis [6] and has been demonstrated to decrease platelet aggregation, blood viscosity, and the risk of both thrombus formation and angina pectoris in various in vitro and in vivo studies [7,8]. Bromelain preparations have also been extensively used in the nutraceutical and cosmeceutical sectors and in the food industry for beer manufacture, meat tenderization, gluten degradation in baking, or improvement of cheese properties [9,10].

Bromelain is present in almost all parts of pineapple (peel, leaves, stem, and fruit) but can be found in the highest amounts in fruit and stems. The pineapple stem primarily provides stem bromelain (EC 3.4.22.32) [1] but also contains other proteinases, including ananain, comosain [11], and acidic stem bromelain [2]. Fruit bromelain (EC 3.4.22.33) is the major proteolytic enzyme in the fruit flesh [12], and two active isoforms of this enzyme have been identified in pineapple fruit crude extracts [13]. Whereas bromelain from pineapple

stems has an isoelectric point (pI) of 9.5 and an optimum pH range of 6–7, the bromelain extracted from the fruit is characterized by a pI of 4.6 and an optimum pH range of 3–8 [2,14]. These differences are most likely due to the presence of different thiol-endopeptidases in bromelain preparations from either the fruit or the stem and most likely also explain the great heterogeneity of clinical and preclinical results for the therapeutic effects of bromelain in the treatment of inflammatory diseases and immune dysfunctions [8].

Since the demand for bromelain has been quickly increasing in recent years due to its broad application as a protease in various industrial fields [15], its recovery from pineapple residues appears as a sustainable and effective waste management strategy. This approach may indeed counterbalance the serious environmental problems raised by the dramatic amounts of waste produced during pineapple fruit processing, transportation, and storage, which are often degraded in landfills, resulting in a dramatic release of harmful greenhouse gases and substances to the environment [16,17]. In this frame, pineapple core is one of the most significant waste materials in the production of canned pineapple, jam, or juice, but it usually goes to landfills or for animal feeding. Pineapple core contains relevant amounts of glucose and fructose that make it suitable as a substrate for microbial fermentation processes aimed at producing organic acids that find applications, among others, in the food industry [18]. The core has been widely used as a source of fibers, phenolics and antioxidant compounds, and vitamins [10] and is generally richer in bromelain than other pineapple residues [19,20]. As for this latter issue, a number of researchers have focused their attention on measuring the proteolytic activity of core-derived bromelain preparations, but specific efforts are required for the molecular identification of the enzymes from the pineapple core [18,20].

In this project, we focused on the preparation and the biochemical characterization of bromelain extracts from either pineapple core or fruit. The application of enzymatic activity tests and specific proteomics approaches allowed the comparison in terms of enzymatic activity and composition of the bromelain preparations obtained from the core or the pulp. In particular, this study will help identify the peptidase(s) extracted from either the pineapple pulp or core and may contribute to addressing the possibility of recovering biologically active bromelain from the core. Taking into account that fruit bromelain can be easily extracted from pineapple pulp juice [9,21], our approach may pave the way to the valorization of this specific waste material and to the application of a circular economy in this specific industrial sector.

2. Materials and Methods

2.1. Preparation of Protein Extracts

Pineapples (*Ananas comosus* (L.) Merr., MD-2 variety) were purchased in a local supermarket, based on size uniformity, at a similar ripening stage, and free of visual defects. The pineapples were washed with water and manually peeled, and the core was mechanically separated from the pulp. The core and the pulp were cooled down at 4 °C, mechanically blended by a lab-scale blender (8010ES two-speed blender, Waring Commercial, Stamford, CT, USA), and eventually centrifuged at 5000× *g* at 4 °C for 20 min to separate the insoluble particles from the juice by using a refrigerated J2-21 centrifuge (Beckman Instruments, Palo Alto, CA, USA). Dry matter was determined on 20 g of blended material, dried to a constant weight at 105 °C overnight in a laboratory dry oven (Falc Instruments, Treviglio, Italy) according to standard requirements (AOAC method 922.10) [22]. The residue was weighed and reported as a percentage of the starting material. Results are expressed as grams of solid matter/100 g of fresh pineapple.

2.2. Protein Quantification and SDS-PAGE

Soluble proteins in the juice from either the pulp or the core were quantified by using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Milan, Italy) and were separated by SDS-PAGE [23]. For protein quantification, 0.01 mL of each juice was used. In the case of SDS-PAGE, samples were diluted at a 1:1 ratio with denaturing

buffer (0.125 M Tris-HCl, pH 6.8; 50% glycerol (*v*/*v*); 17 g/L SDS; 0.1 g/L Bromophenol Blue) in the presence of 2-mercaptoethanol and heated at 100 °C for 5 min. SDS-PAGE was carried out in a MiniProtean Tetra Cell apparatus (Bio-Rad, Milan, Italy) on a 12% acrylamide-bisacrylamide (37.5:1 ratio) gel, using a Tris/glycine buffer system. Gels were stained with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific, Milan, Italy).

2.3. Enzymatic Assay

Bromelain activity was measured by slightly modifying the procedure used by Ketnawa and co-workers [17]. In particular, the juice obtained from either the core or the pulp was centrifuged at 13,000× *g* for 15 min at 4 °C, and 0.02 mL of the supernatant was mixed with 1 mL of 1% (*w*/*w*) sodium caseinate in PBS buffer, pH 7.0, in the presence of 6 mM EDTA and 30 mM cysteine, and incubated at 37 °C for 10 min. After the addition of 0.85 mL of 5% (*w*/*v*) trichloroacetic acid (TCA), the reaction mixture was centrifuged at 10,000× *g* for 10 min. The number of soluble peptides in the supernatant was determined by measuring the absorbance at 280 nm, and the bromelain proteolytic activity was expressed as the amount of enzyme that releases 1 µmol of tyrosine per minute under the assay conditions, considering an extinction coefficient for tyrosine at 280 nm of 1490 M^{-1} cm^{-1}. A blank solution was prepared by mixing the caseinate solution and the various samples and by immediately stopping the reaction by TCA addition.

2.4. Mass Spectrometry

After protein separation by SDS-PAGE, the gel bands were excised and underwent a trypsin in-gel digestion procedure. NanoUPLC-hrMS/MS analyses of the resulting peptides mixtures were carried out on a Q-Exactive orbitrap mass spectrometer (Thermo Fisher Scientific, Milan, Italy), coupled with a nanoUltimate300 UHPLC system (Thermo Fisher Scientific, Milan, Italy). Peptide separation was performed on a capillary easy spray (0.075 mm × 150 mm, 1.7 mm) using aqueous 0.1% formic acid (buffer A) and CH_3CN containing 0.1% formic acid (buffer B) as mobile phases and a linear gradient from 5% to 35% of B in 60 min and a 300 nL/min flow rate. Mass spectra were acquired over a m/z range from 350 to 1500. To achieve protein identification, MS and MS/MS data underwent Mascot software (v2.5, Matrix Science, Boston, MA, USA) analysis using the non-redundant Data Bank UniprotKB/Swiss-Prot (Release 2022_01). Parameter sets were trypsin cleavage; carbamidomethylation of cysteine as a fixed modification and methionine oxidation as a variable modification; a maximum of two missed cleavages; false discovery rate (FDR), calculated by searching the decoy database, 0.05.

2.5. Statistical Analysis

Data presented in Tables 1 and 2 and in Figure 1 are the average of three replicates from three independent measurements and are reported as average ± standard deviation. Analysis of variance (one-way ANOVA) was carried out by using Statgraphic Plus v. 5.1 (StatPoint Inc., Warrenton, VA, USA). Data from MS analysis reported in Table 3 were obtained by performing three replicates from three independent measurements.

Table 1. Dry weight and amount of soluble proteins in the centrifuged extracts of pineapple fruit pulp and core. Amounts of soluble proteins were measured by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Milan, Italy).

Sample	Dry Weight (g/100 g Fresh Product)	Soluble Proteins (mg/g Dry Material)
Pulp	12.0 ± 1.1	15.6 ± 1.0
Core	10.9 ± 0.8	6.8 ± 0.4

Table 2. Enzymatic activity on sodium caseinate of bromelain preparations from pineapple pulp and core. Bromelain proteolytic activity is expressed as the amount of enzymes that release: (i) one µmol of tyrosine per minute from casein under the assay conditions; (ii) one µg of tyrosine equivalents per hour from casein under the assay conditions (papain unit, PU); and (iii) one mg of amino nitrogen from a standard gelatin solution after 20 min of digestion (gelatin digesting unit, GDU).

Sample	Bromelain Enzymatic Activity		
	µmol Tyrosine/mg Protein × min	PU/g Protein ($\times 10^6$)	GDU/g Protein
Pulp	3.32 ± 0.06	36.1 ± 0.7	2407 ± 45
Core	3.38 ± 0.09	36.7 ± 1.0	2446 ± 71

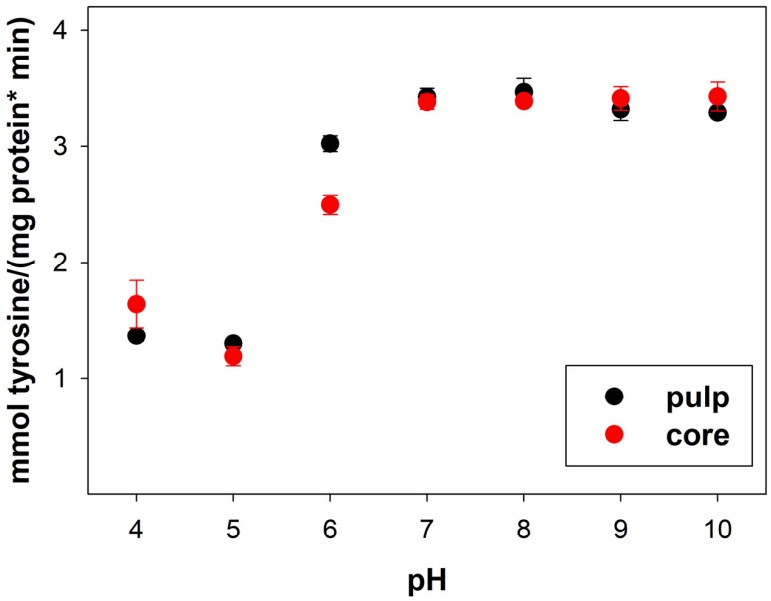

Figure 1. pH dependence of the proteolytic activity on sodium caseinate of bromelain extracts from either pineapple pulp or core.

Table 3. Identification of fruit bromelain (BROM1_ANACO) and stem bromelain (BROM2_ANACO) peptides in the three analyzed samples based on MS/MS data. * These peptides have the same amino acid sequence and cannot be discriminated based on MS/MS data.

Sample	Experimental MW	BROM1 Peptide	Experimental MW	BROM2 Peptide
Pulp	1070.551 2477.145 1540.669	121–129 * 164–185 300–313	1070.550 938.471 1927.008 750.345 1067.565 1124.587 1583.758 950.461	1–9 * 10–18 43–59 65–70 71–79 80–90 98–112 180–187
Core	1070.5506 2477.1453 1540.6688	121–129 * 164–185 300–313	1070.5506 938.4709 2470.1471 1927.0154 750.347 1067.5662 1124.5867 1583.7587 950.4613	1–9 * 10–18 19–40 43–59 65–70 71–79 80–90 98–112 180–187

Table 3. *Cont.*

Sample	Experimental MW	BROM1 Peptide	Experimental MW	BROM2 Peptide
Commercial	1070.5506	121–129 *	1070.5506	1–9 *
	2477.145	164–185	938.4709	10–18
	1540.6665	300–313	1927.0083	43–59
			750.3452	65–70
			1067.5655	71–79
			1124.5893	80–90
			1583.7583	98–112
			1940.9632	128–144
			950.4615	180–187

3. Results

3.1. Sample Preparation

Crude protein extracts were prepared from either pineapple core or pulp after the mechanical separation of the core from the rest of the fruit without adding additional water or any other buffer or solvent for extraction. The core and the pulp were blended and centrifuged to remove insoluble particles. This allowed the recovery of a clear supernatant that contains the soluble bromelain proteinases. Total proteins in the extracts were quantified by the BCA assay (Table 1) and normalized according to the total dry weight of the starting material. Table 1 highlights a total protein content in the pulp more than double that in the core, as already reported in the literature [17]. This difference could be attributed to the specific biological composition of the pulp and the core, the latter being richer in fibers [24].

3.2. SDS-PAGE

Proteins in the extracts were separated by SDS-PAGE that provided very similar tracings for the various samples, with a main protein band at around 23–25 kDa (Figure 2). This indicates that proteins extracted from the fruit flesh and core were almost the same and most likely corresponded to bromelain. In this frame, our results are comparable to those obtained in previous studies, where bromelain from pineapple core and pulp were reported to be 26 kDa and 23 kDa, respectively [2,25]. A commercial bromelain preparation (Merck, Milan, Italy) was used as a reference and showed a slightly different electrophoretic pattern than that of our extracts. In particular, the SDS-PAGE tracing of the commercial preparation is characterized by a main protein band with a molecular weight of ∼25 kDa but also by a number of bands at lower MW that might result from proteolytic degradation. Differences in the electrophoretic pattern might be due to differences in terms of post-translational modifications and/or proteolytic maturation between the proteins present in our extracts and those in the commercial sample. As for these issues, the bromelain source (e.g., fruit, core, stem) and the specific pineapple variety, harvesting, and ripening stage are to be considered [26,27].

SDS-PAGE measurements also highlighted that bromelain is the most abundant protein that could be detected in the various preparations with very few contaminants, thus indicating that its purification from the pineapple core does not require dramatic efforts. Given that the various extracts considered in this study were obtained by an aqueous extraction of the blended fruit pulp or core followed by a centrifugation step, this appears quite relevant from a practical standpoint, with a special focus on the identification of a procedure that would be effective and efficient as for providing bromelain with high yields and purity level, while limiting the production costs.

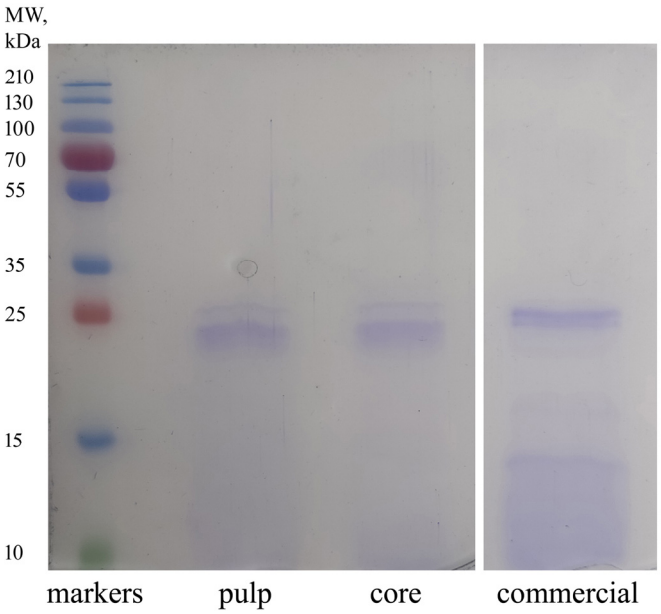

Figure 2. SDS-PAGE of the proteins from the centrifuged extracts of pineapple fruit pulp and core. Proteins were separated on a 12% acrylamide-bisacrylamide (37.5:1 ratio) gel using a Tris/glycine buffer system. Gels were stained with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific, Milan, Italy). A commercial bromelain preparation (Merck, Milan, Italy) was used as a reference and analyzed by SDS-PAGE.

3.3. Mass Spectrometry Identification

To better elucidate the protein pattern in either the pulp or the core extracts, samples underwent an SDS-gel separation of the proteins followed by tryptic in situ digestion and nanoLC-hrMS/MS analysis of the resulting peptide mixtures. The same protocol was also applied to a commercial bromelain preparation, used as a control. For each sample, various bands with apparent molecular weights in the range of 17–25 kDa were excised and subjected to the MS-based identification procedure. Similar results were obtained for all the bands (Table 3), showing that they contain two different proteins: fruit bromelain (BROM1_ANACO) and stem bromelain (BROM2_ANACO). Although the mature form of these two proteins has a 70% sequence identity, there are several tryptic peptides unique to each isoform, which allow the two proteins to be discriminated.

Both electrophoretic and LC-MS/MS analyses suggested that the fruit and the core contain the same proteins. Indeed, the differences observed in the identified peptides may stem from slight variations in the abundances of each species in the gel bands rather than from modifications in the protein sequences. Furthermore, the peptides from the two bromelains identified in the samples were substantially the same as observed for the commercial preparation. The different electrophoretic profiles obtained for the latter sample found no correspondences in the MS data. This might be due to differences in the glycosylation of the proteins in the commercial sample compared to the other samples, although it was not possible to observe the glycopeptides in the LC-MS/MS analyses. As for this issue, it should be emphasized that the purpose of this work was rather to identify and compare by a well-established proteomics protocol the proteins extracted from different parts of the same fruit, and not to demonstrate that the bromelain preparations considered in this study were identical to the marketed one, of which, however, the precise source is ignored.

3.4. Enzymatic Kinetic Measurements

The detection of the enzymatic activity of the various bromelain extracts is essential to measuring their bromelain content, which in turn reveals the yield efficiency of the extraction process, as well as the stability of the proteolytic activity under various

operational and storage conditions (pH, temperature). Different substrates can be used to assess bromelain activity, ranging from casein and gelatin to the more sensitive casein and albumin azo-derivatives and various artificial peptides that release easily detectable molecules following proteolysis [28]. Casein (often in the form of sodium caseinate) is the most frequently used substrate for proteolytic activity measurements [29].

In this work, the extracts from either the pulp or the core were tested for their proteolytic activity on sodium caseinate by spectrophotometrically measuring the amount of released soluble peptides at 280 nm. In this frame, enzymatic kinetics for both preparations appeared comparable, as highlighted in Table 2. Enzymatic activity was also calculated and expressed as papain unit (PU) and gelatin digesting unit (GDU), which are conventionally used to provide bromelain activity of commercial preparations [30]. In particular, one PU is defined as the amount of enzyme that liberates one microgram of tyrosine equivalents per hour from casein under the assay conditions [31], whereas one GDU is the amount of enzyme which liberates 1 mg of amino nitrogen from a standard gelatin solution after 20 min of digestion and conventionally corresponds to ~15,000 PU [31].

Our preparations showed an enzymatic activity comparable to most of the bromelain-based products commercially available in the Italian market (data not shown) but also to that reported in a number of studies that used different physical approaches to obtain bromelain from various pineapple waste materials and by-products [19,32]. These results also confirm that our extraction procedure does not impair bromelain proteolytic activity, although further investigation is required to address potential effects on the enzyme when shifting from a lab-scale to the industrial processing of pineapple.

The effect of pH on the enzymatic activity of the peptidases from the pulp or the core was also addressed, and a similar trend was observed for both preparations that show the highest activity at around pH 7.0, although no significant differences in peptide release were observed in the pH range 7–10 (Figure 1). These results further confirm the presence of very similar (if not the same) peptidases in the pulp and in the core.

As for the pH effect on the activity of bromelain from various pineapple parts, our results are consistent with the observations of Gul and coworkers [33], although other studies highlighted a relevant decrease of the proteolytic activity of fruit bromelain on sodium caseinate at pH higher than 8 [19,29]. This behavior may be explained by differences in terms of bromelain source and of variety, harvesting, and ripening stage of the pineapples used in the various studies [17].

4. Discussion

Pineapple wastes are generated from the processing of juice, canned, and frozen products, but also from the harvesting and processing practices, and can account for 50% of pineapple weight, and their disposal represents a dramatic issue that may be related to serious environmental problems [18]. In this frame, an effective waste management strategy may involve (i) the use of waste as a substrate for bacterial growth and the biotechnological production of ethanol, citric acid, and antioxidant compounds and ii) the recovery of added-value products that include cellulose, hemicellulose, and other carbohydrates, and various enzymes, among which bromelain is the most representative and commercially-valuable (its cost can range up to 2400 USD/kg) [17,19].

In the present work, we addressed the extraction and enzymatic characterization of bromelain from the pineapple core, which is one of the most significant waste materials in the production of canned pineapple, but usually goes to landfills or is used for animal feeding. Pineapple core is a relevant bromelain source since it is generally richer in bromelain than other pineapple residues and represents around 15% of the total processing waste [19].

In this work, bromelain was extracted from either pineapple fruit pulp or core, and the comparison of the two preparations showed significant differences in the protein content due to their specific biological composition and the higher amount of fibers in the core (Table 1). However, no differences could be detected for the specific proteolytic activity on caseins by the two different preparations (Table 2) and for the effect of pH on their

enzymatic activity (Figure 1). Similar observations stem from the SDS-PAGE separation of the proteins in the extracts (Figure 2) and from their MS identification (Table 3). This latter result appears dramatically relevant since it confirms that the same peptidases are present both in the core and in the pulp, where we were able to identify peptides from both the fruit and the stem bromelain. This confirms the possibility of using pineapple core to obtain relevant amounts of biologically active bromelain since it is fully comparable—if not the same—to the enzyme obtained from the pulp, which is nowadays the primary source of industrial fruit bromelain [21].

In our study, highly pure bromelain preparations were obtained by applying a relatively simple procedure that only included homogenization and centrifugation of the pineapple core. In this frame, it has to be highlighted that several limitations, including temperature- or process-induced inactivation and/or aggregation, still impair the efficiency and the effectiveness of bromelain recovery from pineapple extracts, although novel purification strategies and biotechnological processes have been applied to mitigate production costs [8].

Production costs strongly depend on the purification process applied to obtain commercial bromelain at the desired purity level, which may involve ammonium sulphate precipitation, an aqueous two-phase system, membrane filtration, and various chromatographic approaches (ion exchange and/or size-exclusion) [18,34]. However, the direct comparison of the various bromelain extraction and purification techniques reported in the literature is not a straightforward task because of the wide heterogeneity in terms of bromelain source, extraction strategies, enzymatic activity test conditions, and ways of expressing bromelain activity [8]. The selection of an effective purification procedure should take into account the feasibility, ease of scale-up, and the cost of the specific approach, but also the commercial fate of the bromelain, considering that most of the bromelain applications do not require an enzyme completely free of contaminants.

The use of a simplified and mild procedure—as the one carried out in this project—may allow a decrease in the overall production costs for bromelain production and further contribute to its use in nutraceutical and pharmaceutical preparations or in various industrial, technological processes [10,35]. Further efforts are surely required for the successful shift from the laboratory to the commercial scale of the process considered in this study, but our approach is paving the way to the valorization of pineapple core as a relevant bromelain source, which provides the same enzyme as the one obtained from the pineapple fruit juice. The implementation of a circular economy in this specific industrial sector would allow the use as a valuable resource of the biomass associated with (and resulting as a waste material from) pineapple production and processing and its conversion into high-value-added products, thus contributing to improved societal economics. This approach may lead to the development of more sustainable and innovative industrial technologies and to a zero-waste scenario [36,37], both of which represent highly relevant issues for the food industry that need immediate action [16].

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