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Pectins

The New-Old Polysaccharides

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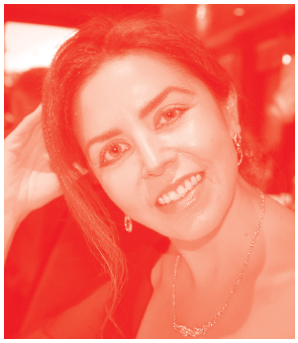

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Martin A. Masuelli is a Inv. Adj. professor at the Instituto de Física Aplicada, National Scientific and Technical Research Council (CONICET), and an associate professor at the National University of San Luis (UNSL), Argentina. He holds a master's degree and a Ph.D. in Membrane Technology from UNSL. He has served as the director of the Physics Chemistry Service Laboratory, UNSL, since 2014. He is an expert in polysaccharides and the physical chemistry of macromolecules. Dr. Masuelli has authored or co-authored more than thirty-two peer-reviewed international publications, eight book chapters, and seventy communications in international congresses. He has also edited seven books. He is a member of the Sociedad Argentina de Ciencia y Tecnología Ambiental, Asociación Argentina de Fisicoquímica y Química Inorgánica, and Asociación Argentina de Tecnólogos de Alimentos. He is editor in chief and founder of the Journal of Polymer and Biopolymers Physics Chemistry and an editorial board member for various other journals. His research interests include hydropolymers, biopolymers (separative, purification processes, and characterization), physiochemistry of macromolecules, membrane technology and design (NF-UF-MF), and separative processes.

Contents

Preface

Pectin is an industrial product of certain fruit peels that contain it, such as citrus fruits, apples, pears, grapes, plums, beets, sunflowers, and so on. Pectins are a specific group of carbohydrate polymers composed largely of polygalacturonic acid units, specifically polysaccharides, which are part of the larger class of plant-derived pectic substances. Typical raw materials are apple pulp and citrus peel, from which pectin is obtained by acid/base hydrolysis and precipitation using alcohols or aluminum salts. Pectin is the traditional gelling agent for jams and jellies, but its applications extend to fruit products for food, dairy, dessert, soft drink, pharmaceutical, and other industries.

This book consists of three sections and eight chapters.

Section 1: "Pectin Production" includes Chapter 1: "Production of Pectin from Citrus Residues: Process Alternatives and Insights on Its Integration under the Biorefinery Concept" and Chapter 2: "Sustainable Horticultural Waste Management: Industrial and Environmental Perspective."

Section 2: "Pectin Biotechnology" includes Chapter 3: "Biotechnology Applications in Pectin Industries"; Chapter 4: "Fungal Pectinases in Food Technology"; and Chapter 5: "The Microbial Degradation for Pectin."

Section 3: "Pectin Applications" includes Chapter 6: "Pectin-Based Scaffolds for Tissue Engineering Applications"; Chapter 7: "Pharmaceutical Applications of Pectin"; and Chapter 8: "Effect of Cross-Linking Agent on Mechanical and Permeation Properties of Criolla Orange Pectin."

This book is for students, professionals in the food and pharmaceutical industries, and researchers studying pectins and biopolymers.

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Section 1

Pectin Production

Chapter 1

Production of Pectin from Citrus Residues: Process Alternatives and Insights on Its Integration under the Biorefinery Concept

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Abstract

This chapter describes the pectin production process from citrus residues. It discusses the importance of essential oils removal before processing through steam distillation, hydrodistillation, or solvent extraction. Also, it presents different extraction methods (acid hydrolysis, microwave-assisted acid hydrolysis, and hydrodistillation) that have been employed and different solvents that can be used for its purification. Since all these processing parameters can affect the final pectin yield and quality, a discussion is made on which processing options and conditions could be used based on recently reported data. The best operational conditions based on the percentages of pectin recovery and their relationship with quality parameters, such as the galacturonic acid content and degree of esterification are presented. Finally, a discussion is made regarding the opportunities for its integration under the biorefinery concept that could help to enhance several economic and environmental aspects of the process.

Keywords: pectin, citrus residues, bioactive compounds, biorefinery, sustainability

1. Introduction

Nowadays, residues are wrongfully disposed of and underutilized, becoming an increasingly alarming problem for the environment and the population's well-being. One of the primary sources of waste is the food industry. It is estimated that about 1600 Mton of food residues are produced annually, and about 500 Mton are entirely derived from fruits [1]. The consumption of natural fruit juices has been increasing recently, mainly due to health concerns in the population. A shift toward a healthier and more natural lifestyle implies a reduction in the intake of soft drinks that could contain a high concentration of sugars, artificial colorants, and artificial sweeteners with possible adverse effects on the human body [2]. Orange juice holds most of the market share due to its vitamin content and general health benefits. As with other citrus fruits, the majority of the fruit is discarded during the juice-making process.

The residues include peels, seeds, and remnant pulp, which represent almost 50% of the total weight of the fruit [3].

Over the years, research has been made to develop ways to use organic waste as a source of chemical substances and energy. There are many studies regarding the obtention of multiple products from citrus peels [1, 4–6]. Some of these added-value products include pectin, essential oils, bioethanol, biogas, and polyphenolic compounds. These products can serve as feedstocks for other industrial processes or as final products by themselves, so the possibilities for selling them are very extensive.

Nonetheless, pectin has been one of the main chemical substances retrieved from citrus residues with organoleptic characteristics that depend highly on the processing steps and conditions used for its production. Moreover, due to the multiple value-added products obtained from citrus residues, it is appealing to investigate the possibility of integrating all these processes under the biorefinery concept, which encompasses a series of steps aimed to transform, refine, purify, or separate different kinds of biological assets into other products [7].

This chapter intends to compile relevant information regarding the production of pectin from citrus residues and thus, determine the most efficient methods that result in the best quality and yields of the final product. Using information collected in the last ten years and reported in relevant scientific databases (Scopus, Springer Link, Wiley, Taylor & Francis, and ACS), a description of the processing alternatives for pectin production was made. Additionally, the gathered information was used to propose the most convenient alternatives and process conditions for its obtention. Finally, the possibility of integrating pectin production into a whole citrus residues biorefinery was discussed, including novel valorization pathways that could increase the process's economic, environmental, and social sustainability.

2. Unit operations and process conditions for pectin extraction from citrus residues

In the last few years, studies on developing new routes for utilizing organic citrus residues have mainly focused on pectin production. Pectin is primarily found as a component of the cell wall of plants that gives them resistance and flexibility due to its content of galacturonic acid, partially esterified with methyl ester or acetyl groups [8]. In general, the process begins by collecting citrus residues. The raw material is then washed, dried, and grinded before bioactive compound extraction. During the extraction of bio-compounds, essential oils, polyphenols, and flavonoids are removed to improve pectin's quality. After this step, pectin is retrieved from biomass by breaking down the polymer and "dissolving it" into the liquid phase. The solid phase residue contains other structural carbohydrates that could be further valorized. The liquid, rich in galacturonic acid units, is then submitted to a separation step ("precipitation"), where it is washed with alcohols or organic solvents that cause pectin to agglomerate. These solvents also eliminate remnant bioactive compounds that can alter the final pectin's organoleptic properties. Finally, solvents are evaporated from the jellified pectin to obtain the product of interest. **Figure 1** shows a diagram representing each one of the processing steps to obtain pectin.

2.1 Preparation of the material

As seen in **Figure 1**, the process begins by washing the material to eliminate excess dirt. After that, citrus residues are prepared for further processing by drying,

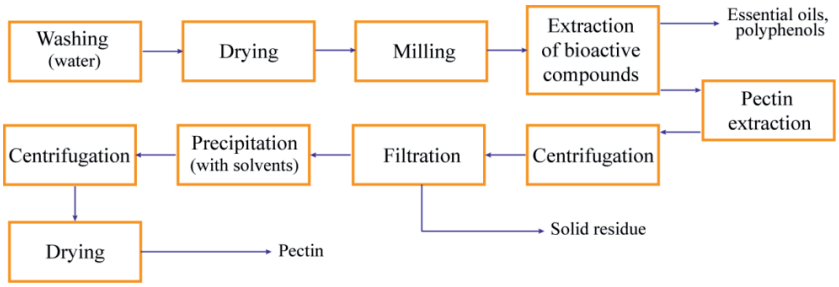

Figure 1.
Process block diagram representing the unit operations to produce pectin from orange residues.

which guarantees their storage for long periods. The material's drying process is usually carried out at temperatures around 40–60°C and drying times up to 2 days. However, the highest drying temperature reported is 95°C [9], which reduces the drying time but could cause the degradation of bioactive compounds. Also, it is desired to achieve low humidity (approximately 10%) as a way to extend the storage time of the raw material and to achieve a small particle size (< 1 mm) that generates a higher contact surface and a better performance during extraction [9].

2.2 Extraction of essential oils and bioactive compounds

It is important to remove certain bioactive compounds such as essential oils and flavonoids, besides some sugars interfering with the pectin's final quality. The purpose of removing these compounds is to improve pectin's esterification degree, galacturonic acid content and guarantee its physicochemical characteristics. At this stage, the principal compound of interest is the essential oil coming from the flavedo of the citrus peel. The essential oils from citrus fruits are conformed mostly by terpenes, which are organic substances responsible for the vegetal material's organoleptic properties. With terpenes removal, unpleasant flavors are avoided, which improves the quality of the final product [10].

Multiple methods such as vapor explosion, hydrodistillation, steam distillation, and in some cases solvent extraction can be implemented to perform essential oil extraction. The most common method used is steam distillation. In this method, the organic material is placed in a container where steam can pass and reach the sample uniformly. On the other hand, hydrodistillation works by placing the residue in direct contact with boiling water. The essential oils are retrieved once the water vapor rich in terpenes and terpenoids is condensed in both cases. Nonetheless, hydrodistillation can present agglomerations due to the direct contact of the submerged material with the liquid, which interferes with steam access to specific system zones. Another extraction method is Solid–Liquid Extraction, which can be done with various polar and non-polar solvents to retrieve the bioactive compounds selectively. However, SLE can also be assisted by heat, agitation, ultrasound, or microwaves, increasing the yields of the desired compounds.

In **Figures 2** and **3**, the yields of essential oils and the limonene content reported using different extraction methods for orange residues are shown in relationship with the pectin process. In **Figure 2**, the highest essential oil yields were obtained using Solid–Liquid Extraction with acetone (~2.2%) [1]. Nonetheless, the Solid–Liquid Extraction with acetone would require further separation of the polar and non-polar compounds due to the polarity of the solvent. For steam distillation, yields of 0.7% [11] and 0.84% [9] were obtained, which are slightly lower than those obtained by Hilali et al. with hydrodistillation and solar hydrodistillation ~1% [12]. Differences observed in yields for steam distillation could be attributed to the

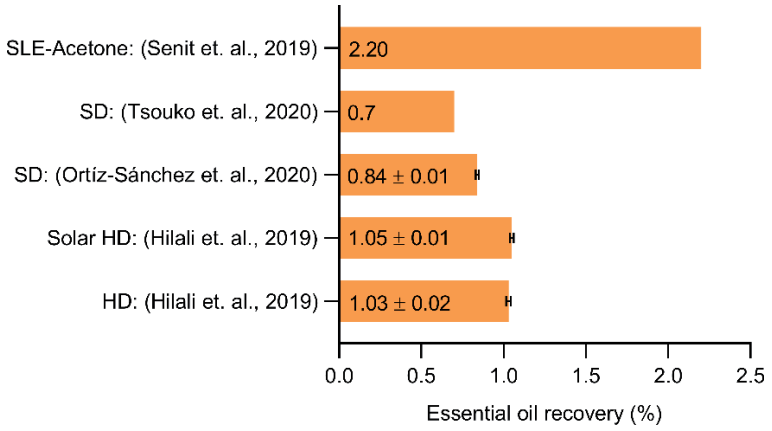

Figure 2.
Essential oil recovery.

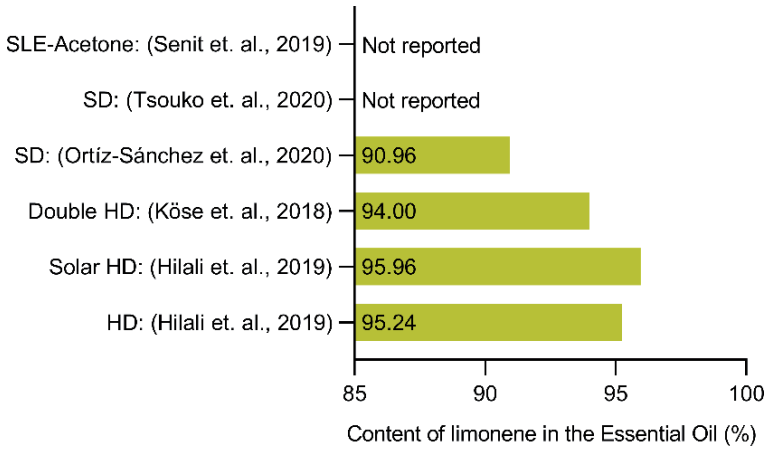

Figure 3.
Content of limonene in the essential oils extracted.

distribution of the sample in the system and how steam interacts with the residue. It is possible to increase steam distillation yields by increasing the pressure in the system (steam explosion) or performing double hydrodistillation [10, 13]. In the case of hydrodistillation, similar yields were obtained independently if the process is carried out with solar energy or not. As seen in **Figure 3**, the limonene content in the essential oils of orange residues is between 90% and 95% [9, 10, 12].

2.3 Extraction of pectin

Once essential oils and other bioactive compounds are removed, the extraction of pectin can be carried out. The first option is to use the liquid phase from hydrodistillation, rich in pectic substances released during heating in direct contact with water. Since pectin is heat-sensible and water-soluble, this option is attractive to perform both essential oils removal and pectin extraction. Hilali et al. reported a yield of ~12% for conventional hydrodistillation and ~ 8.3% for solar hydrodistillation [12]. Even though similar yields were obtained for essential oils using hydrodistillation, the way the heat is applied to the system may affect how much of the pectin is dissolved, resulting in lower yields. Similar behavior can be observed when pectin is retrieved from microwave-assisted hydrodistillation, with yields of around 15% [14].

The most common way to extract pectin from citrus residues is to employ acid hydrolysis, which consists of breaking down the bonds of pectin to obtain galacturonic acid units at high temperatures (from 80–116°C) and low pH values (1–3) with the help of dilute inorganic or organic acids. These hydrolysis reactions can also be assisted by agitation, which enhances the rate of depolymerization of pectin. **Figure 4** shows the best yields reported in recent literature for the pectin extraction process using different acids and processing conditions. From the inorganic acids in **Figure 4**, the highest yields were obtained using sulfuric acid (30.5%) [15], phosphoric acid (29.4%) [16], and hydrochloric acid with (~25%) [11]. It is important to note that the hydrolysis performed with sulfuric acid was completed at shorter times and higher temperatures (10 min and 116°C) [15] than the ones done with phosphoric acid (120 min, and 95°C) [16].

Moreover, the similar yields of pectin obtained from citrus residues using hydrochloric acid with different processing times [11, 17, 18] allow us to hypothesize that longer times could only cause a slight increase in the yield of pectin when temperatures are higher than 95°C at low pH values (1.6–1.8). On the contrary, lower temperatures (around 80°C) with hydrochloric acid reduce pectin yields. As seen in **Figure 4**, pectin yields decreased down to 16–20% [1, 19]. On the other hand, the hydrolysis of citrus residues using organic acids is mainly done with citric acid. The highest pectin yield reported using citric acid is 32.6% (160 min, at 90°C, and pH 2) [11], attributed to the long hydrolysis time. In **Figure 4**, it is possible to see that a short time of hydrolysis with citric acid results in lower yields. Once again, the use of temperatures around 80°C decreases pectin yields considerably, a behavior that was also observed when using inorganic acids. In the work of Rodsamran et al., microwave-assisted acid hydrolysis of lime residues was performed, with yields of ~16% and ~ 10% of pectin, for hydrochloric acid and citric acid, respectively [18];

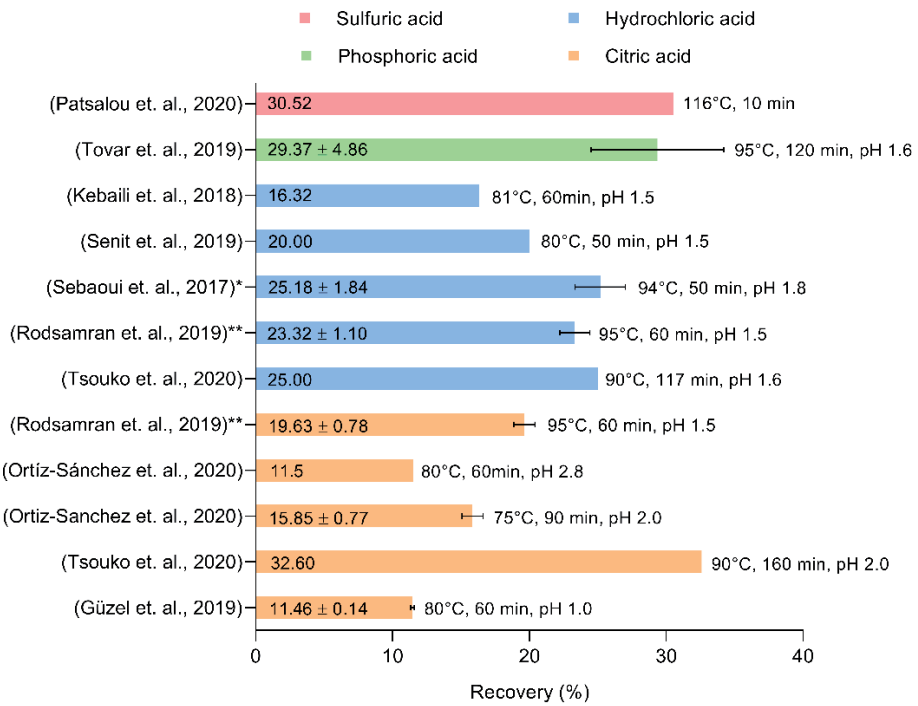

Figure 4.
*Yield of pectin obtained from acid hydrolysis of citrus residues (Orange peel, *lemon peel, **lime peel) using sulfuric acid, phosphoric acid, hydrochloric acid, and citric acid.*

once again, the yields obtained with the inorganic acid resulted higher. The implementation of microwave-assisted hydrolysis has the benefit of implementing shorter process times (~5 min) but has the disadvantage of altering the final color of pectin, making it more brownish than the desired one for commercial pectin [18].

The reported data in **Figure 4** shows that the use of strong acids results in a better hydrolysis performance than organic acids due to their affinity for Ca^{2+} ions, which are responsible for stabilizing pectin chains [18]. However, it has been evidenced that the use of strong acids could be problematic since it causes the loss of some volatile compounds, environmental impacts such as the acidification of rain and water sources [20], and the degradation of valuable remnant substances that could have been further valorized due to their over hydrolysis. Conversely, the use of citric acid may cause lower environmental impacts than those resulting from the use of inorganic acids in the process. In addition, citric acid has been reported to cause less harsh depolymerization of pectin [18]. Also, it is easier to handle its traces during food formulations in comparison to inorganic acids.

2.4 Purification of pectin

The liquid phase that results from the hydrolysis, rich in galacturonic acid, is then retrieved and mixed with alcohols such as ethanol, methanol, 1-propanol, or its isomer isopropanol to separate pectin due to its insolubility in this type of solvents [21]. Most of the authors highlight the use of ethanol, acidified ethanol, or acetone to precipitate citrus pectin. Precipitation of pectin with ethanol is mainly done at 20–25°C, leaving the samples overnight (18 - 24 h) [17, 18, 22]. Depending on the degree of purification desired, different concentrations of ethanol can be used. At least one wash with ethanol at 96% (v/v) is made after pectin extraction. What is more, there are some cases in which the sample is washed three times or more with ethanol at different concentrations (50%, 70%, and 96%), not only to separate pectin but also to remove sugars, polyphenols, and essential oils that remain [1, 8–10, 16, 17, 22, 23]. The removal of these undesired substances helps to obtain pectin in its whitened form. In addition, ethanol could be ideal since it avoids the precipitation of other non-desired compounds [24] and can absorb water from the pectin. Ethanol could also be beneficial for the process since it can be further recovered and reused.

Moreover, since pectin requires acidic conditions for its precipitation, it is necessary to use acidified ethanol (0.5% HCl) when pectin is obtained from hot water extraction [10], as happens when doing hydrodistillation. It is also possible to remove other remnant substances from pectin and increase the organoleptic characteristic of the final product by using a final wash with acetone. For example, Rodsamran et al. used three ethanol washes and a final acetone wash to guarantee almost a complete removal of bioactive compounds and increase the purity of pectin [18].

At this point, some authors report the use of centrifugation to facilitate the separation of pectin from the solvents once they had made effect. Centrifugation has been carried out at low temperatures (4–10°C) using speeds from 4000 rpm to 9000 rpm in a time range of 10 to 20 min [9, 11–13, 22, 23]. After pectin is fully separated, it can be dried at low temperatures that guarantee the thermal stability of the polymer. It is possible to used use vacuum drying at 40°C for short periods of time (1-2 h) [1, 11, 16, 19] or convection drying at 50–55°C for 16 to 24 h [8, 9, 12, 15, 17, 18, 22, 23, 25]. It is important to highlight that pectin yields are primarily affected by other process stages, not by the drying step. However, to guarantee pectin's quality, it is recommended to avoid the exposure of the material to high temperatures for long periods.

2.5 Quality parameters of the final product

To evaluate the final quality of the obtained pectin after purification, the galacturonic acid content and the degree of esterification are the two main characteristics that should always be considered. The galacturonic acid content reveals how much of the retrieved sample contains the primary units to form the polymer. The degree of esterification describes how many carboxyl groups of the galacturonic acid in pectin are esterified with methanol which influences the gelling capacity of pectin. Consequently, both properties help to define the most suitable applications for the extracted pectin.

As can be seen in **Figures 5** and **6**, the highest content of galacturonic acid (~90%) and esterification degree (71–85.6%) was reported by Rodsamran et al. using hydrochloric acid and citric acid in the hydrolysis of lime peels [18]. The standalone result for the esterification degree of orange pectin obtained with phosphoric acid is also high (83.6%) [16] and suggests the necessity of further investigation of the use of this acid in the process. In orange peels, even though broad ranges of galacturonic acid content (50–75%) were reported for hydrochloric acid and citric acid, the esterification degree reported maintained a value around 65–70%. The low galacturonic acid content reported in some cases could be attributed to how the sample was washed to remove remnant phytochemicals and sugars and to the prolonged effect of temperature at low pH values. The decrease in the pH at high temperatures over long periods causes an increment in the degree of dissociation of the carboxylic acid groups [24], leading to the degradation of pectin into substances of lower molecular weight, which ethanol cannot precipitate [26].

It is possible to infer that orange pectin would have similar gelling properties no matter if it were obtained using either citric acid or hydrochloric acid at different process conditions. Since the galacturonic acid content reported in **Figure 5** is always higher than 50% and the esterification degree higher than 65%, it is possible to say that the obtained citrus pectin can be considered as high-methoxyl pectin [27, 28]. This kind of pectin forms its structure based on hydrogen bonds between hydroxyl groups, where sugars, thanks to their highly hydrophilic effect, allow the bonding between polymer chains. High-methoxyl pectin can achieve jellification in few minutes at temperatures around 95°C, suggesting the possibility of using citrus pectin in various food products. On the contrary, low-methoxyl pectin requires

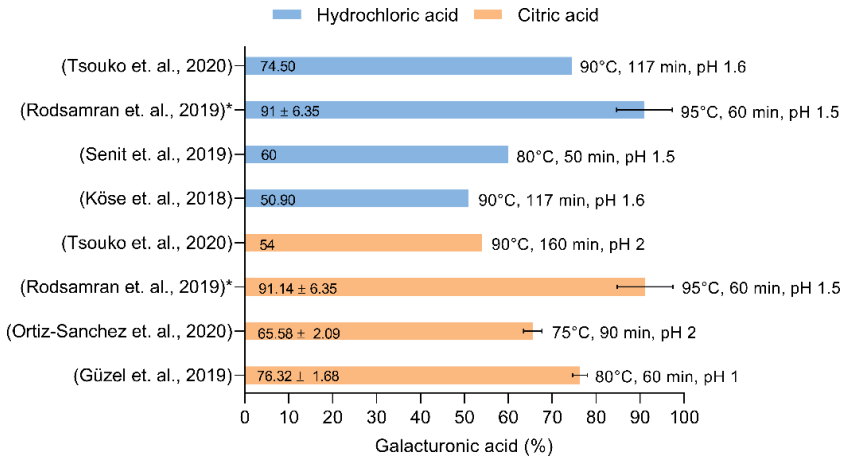

Figure 5.
*Galacturonic acid content of pectin obtained from acid hydrolysis of citrus residues (Orange peel and *lime peel) using hydrochloric acid and citric acid.*

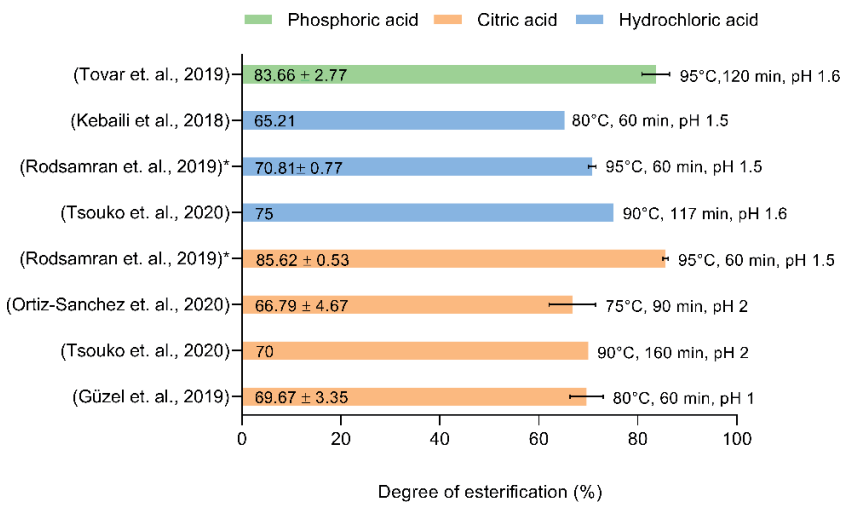

Figure 6.
*Degree of esterification of pectin obtained from acid hydrolysis of citrus residues (Orange peel and *lime peel) using phosphoric acid, hydrochloric acid, and citric acid.*

metallic cations (Ca_2^+ or Mg^{2+}) that bond between themselves and the anionic structure of pectin to form gels due to its low degree of esterification [14].

2.6 Process conditions that enhance pectin quality and recovery

Figure 7 shows a process diagram that suggests the most appropriate process conditions to obtain citrus pectin. In the first place, the raw material must be adequately dried to assure its preservation and milled to increase the contact surface which yields during essential oils extraction and hydrolysis. Secondly, steam distillation is preferable for essential oils extraction since it would selectively retrieve these valuable substances without affecting the material. Contrary to this, during hydrodistillation, the material is in direct contact with hot water, which causes its partial hydrolysis and the degradation of pectic substances, resulting in lower pectin yields; additionally, the use of hydrodistillation would require the acidification of ethanol during precipitation. Thirdly, the acid hydrolysis of pectin can be carried out either with hydrochloric acid or citric acid since the final pectin would always have high-methoxyl properties. Nonetheless, process conditions that tend to increase yields and galacturonic acid percentage should be employed. It is necessary

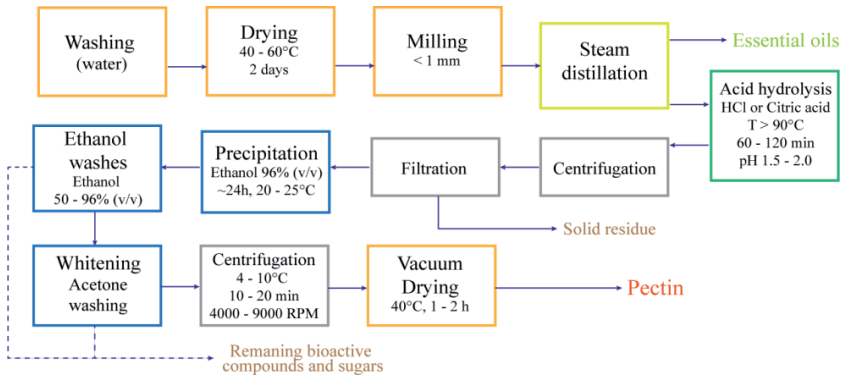

Figure 7.
The production process of citrus pectin and suggested operational conditions.

to perform a careful separation and purification during the final steps to assure high yields and purity of pectin. The last stage of pectin production will always require ethanol at 96% (v/v) for its precipitation and several washes with ethanol and acetone that remove sugars and bioactive compounds. After that, centrifugation is used to assure proper separation from the solvent (that can be later evaporated and reused) and vacuum drying to avoid the degradation of the final product. It is important to highlight that it is possible to obtain additional valuable products from the bioactive compounds extracted through steam distillation and the solids retrieved after hydrolysis rich in lignocellulose.

3. Integration of the process for pectin extraction under the biorefinery concept

It is useful to study how different processes can be integrated with the existent pectin production process under the biorefinery concept to improve the integral sustainability of the valorization of citrus residues. This means that the sustainable use of citrus residues implies the maximization of possible products and energy obtained from this feedstock. For that, it is crucial to consider a logical order in which the different compounds are extracted or produced, as the presence of some of them can impact the quality of other compounds later in the process, which relates to the concept of biomass cascading applied to the biorefinery design process [29, 30]. Additionally, other reagents used along the steps should be carefully selected and studied as they may impact the desired product itself, cause environmental issues, or affect the economic viability of the whole process. Finally, the technical aspects of each step should always be considered to guarantee the quality and yield of the different products.

In this context, citrus residues constitute the primary raw material derived from biomass, and the different processes discussed earlier help to separate it and transform said reagents into chemical substances that can be used as final bioproducts. Nonetheless, there are opportunities to produce more value-added products by integrating the pectin production process with several configurations of other technologies, which are summarized in **Figure 8**. For example, Hilali et al. proposed an orange peel biorefinery that obtains essential oils and pectin but extracts additional value from the solar hydrodistillation process by retrieving partially solubilized polyphenols (flavanones) such as Narirutin and Hesperidin [12]. In another work, Budarin et al. proposed the use of microwave-assisted steam distillation (using only the water present in the peel) and microwave-assisted hydrothermal treatment to obtain essential oils, pectin but also hydroxymethylfurfural and 5-chloromethyl furfural (CMF) which can be used as platform chemicals to produce herbicides, insecticides, pharmaceuticals, monomers, solvents and fuels [31]. Ortiz-Sanchez et al. proposed the anaerobic digestion of the solid residue obtained after acid hydrolysis to produce biogas with a high methane content [9], and also the use of hydrolyzed pectin in a fermentation process with fungi (*T. reesei*) to produce mucic acid [23]. Hydrolysates from orange peel have also been evaluated for their potential to produce other organic acids, such as succinic acid, with the help of fermenting bacteria [15, 32]. Kyriakou et al. extracted more value from orange residues by including an enzymatic hydrolysis step to the solid residue left after pectin extraction to obtain sugars that can be fermented into ethanol and produce biogas from the solid residue from the enzymatic hydrolysis [33]. The fermentation of enzymatic citrus hydrolysates using cellulose-producing bacteria has also been reported [11]. Lohrasbi et al. proposed a variation of the process by first implementing the hydrolysis and then retrieving the essential oils using a flash separator; the solid

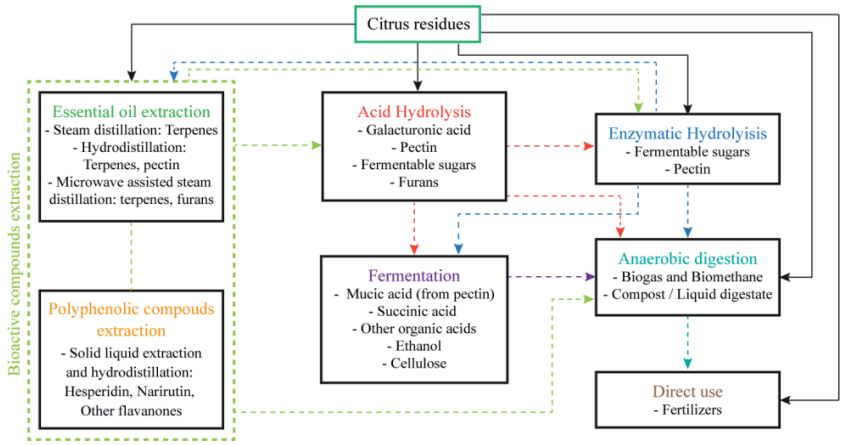

Figure 8.
Alternatives for the integration of the pectin production process under the biorefinery concept.

residue is also used here to produce purified methane, and the digestate obtained from anaerobic digestion is further valorized into compost [34]. As a final option, it has been mentioned that residues from an orange waste biorefinery can be used directly as fertilizers [15].

The biorefinery concept can be associated with several relevant terms such as bioeconomy, circular economy, and industrial symbiosis. Many countries have started promoting policies and programs regarding the bioeconomy as a sustainable development strategy [7]. Circular economy and industrial symbiosis have also gained popularity among the policymakers and stakeholders of different companies. Generally speaking, these three concepts can be summarized as approaches that include the use of biomass-derived feedstocks obtained from various processes from different industries and that contribute to closing down the cycle of industrial processes by using one industry's residues as the feedstocks for another. Not only the value-added products are being produced, but a significant quantity of residues could be used as raw material, a material that would typically end up in a landfill with no further treatment. With this in mind, it is clear why incorporating the processes described above under the biorefinery concept results in a relevant field of study for the valorization of citrus residues and the sustainability of pectin production.

More studies must be performed to determine the feasibility of integrating the possible biorefinery configurations shown in **Figure 8**, the most convenient processing scale [4], and their sustainability. It would be interesting to include not only technical but also environmental, economic, and social aspects into the evaluation of the sustainability of biorefineries from citrus residues by performing an Early-Stage assessment, a methodology that allows the evaluation of multiple biorefinery pathways without the need for vast amounts of data [35–38]. However, the integrated biorefinery's isolated technical, economic, and environmental viability analysis is not enough. It is also essential to demonstrate the sustainability of those bio-based products to promote the deployment of a circular bio-based economy [39] because using residues as feedstocks does not necessarily mean that a process is sustainable. Additionally, in terms of industrial symbiosis, several strategic alliances could be built by selling some of the obtained added-value products to companies that use them as feedstocks. For example, essential oils and polyphenols are mainly used in cosmetics, toiletries, and fragrances due to their essence and benefits for the skin. Also, the market has seen a shift toward organic and natural products,

increasing the popularity of essential oils both in pure form and as additives in skin care and hair products. Other products formulated using biorefinery products are jellies, jams, and frozen foods using pectin. In addition, pectin is widely used in the pharmaceutical industry to reduce blood cholesterol levels and treat gastrointestinal disorders [40]. Other applications include paper substitutes, foams, and plasticizers. Knowing this, the potential benefits of the biorefinery increase, as it would not only align with the current strategies for developing a greener industry, but other companies would also benefit from the possible sustainable-produced chemical substances, materials, and energy derived from the pectin production process.

4. Conclusions

After studying the different options available for pectin extraction, some key findings were made. First, it is crucial to remove essential oils and bioactive compounds beforehand, as they can interfere with the yield and quality of pectin. Citrus essential oil is most commonly removed by steam distillation. However, hydrodistillation and Solid–Liquid Extraction have been shown as an alternative. One advantage of hydrodistillation is that it can also partially extract pectin while the essential oil is retrieved, thus reducing time and resources. Pectin is mainly obtained through acid hydrolysis using different solvents. Hydrochloric acid and citric acid have shown better yields than other solvents, and both result in the obtention of high-methoxyl pectin with rapid jellification. However, when considering an industrial approach, the environmental and safety hazards should be revised; because of this, citric acid represents a better option. It is essential to perform a careful separation and purification of pectin with ethanol and acetone to achieve the appropriate organoleptic properties of citrus pectin. Finally, when considering a biorefinery approach, other valorization alternatives such as the recuperation of flavonoids, the use of sugar-rich hydrolysates to produce ethanol, organic acids, and cellulose, the anaerobic digestion to produce biogas and liquid digestate, and the possibility to use citrus residues directly as fertilizers, are presented as novel possibilities to improve the pectin production process under the biorefinery concept.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Sustainable Horticultural Waste Management: Industrial and Environmental Perspective

Jahidul Hassan, Md. Noor-E-Azam Khan, Md. Mijanur Rahman Rajib, Maksuratun Nahar Suborna, Jiasmin Akter and Md. Faisal Ahamed Hasan

Abstract

Horticultural crops are highly nutritious and shared lion portion of our daily diet. These items are consumed in different ways according to their nature and processing processes. These days, a crucial concerning issue is arising globally to ensure nutrition security for huge population that leads to focus on production increase, quality improvement, food safety assurance, and processing strategies. Consequently, a large amount of waste generates in the processing industries, household kitchen, and supply chain of horticultural commodities that has led to a significant nutrition and economic loss, consequently creating environment pollution with extensive burden of landfills. However, these wastes showed magnificent potentiality of re-utilization in several industries owing to as rich source of different bioactive compounds and phytochemicals. Therefore, sustainable extraction methods and utilization strategies deserve the extensive investigations. This review paper extensively illustrates the horticultural waste generation options, sustainable recycling strategies, and potentiality of recycled products in different industries for betterment in population with the assurance of green environment and sustainable ecology.

Keywords: horticultural waste, management, sustainable, environment, bioactive compounds, pectin

1. Introduction

Nowadays, the horticultural field is exploring with its various utilization. An ever-demanding market is going on with its various options. Vast cultivation in field level with fabulous export potential makes the horticultural product market more outstanding. The global horticultural market value about 20.77 billion USD was estimated in 2021 and targeted to 40.24 billion USD till 2026 [1]. From the kitchen to processing industry, the uses of horticultural product are remarkable today. With its flexibility of uses, problems also arise with various means. The by-products or wastes in horticultural point of view is getting worst day by day for lack of proper utilization not taken. Both developing and developed countries such as Bangladesh, Cambodia, India, Indonesia, Malaysia, Philippines, Thailand, and Vietnam are

suffering from various environmental pollutions in concern with water, soil, and air pollution. The increasing trend of population is found to be the major cause of waste generation [2]. Increased population make wastes more usual in homestead generally produced by unnecessarily.

From the production of horticultural foods, various factors involve for the hazardous environmental appearance. Uncontrolled uses of pesticide and residual effects of various chemicals initiate the primary threats to nature by creating bad impact on the wildlife, soil, human, and animal communities [3]. Sometimes these chemicals (about 5–15%) that introduced in the global market for field management are counterfeit in nature [4]. The food wastes evolved in kitchen is noteworthy in many countries such as China, containing solid food wastes between 88% and 94% [5]. Processing industries greatly influence the environment pollution as so many by-products discarded to the environment from this sector while these by-products contain some high quantities of phytochemicals that can be reusable enough for the better disposal [6]. Massive climate issues arise from the undisposed waste available in the environment [7]. Greenhouse gases increases are also introduced in nature by the inappropriate waste disposal method. So, proper and alternative process of waste disposal is compulsory for economic viability and environmental stability.

Increasing the recycling and developing various disposal methods can ensure the proper mitigation of environmental pollution. The industrial and various sources of horticultural wastes are getting importance for its valuable compositions. Recycling can develop the new opportunities with commercial benefits. Biofuels, enzymes, vitamins, antioxidants, and various important chemicals are manufactured from the industrial wastes today. Waste to wealth can be the modern thought of waste disposal. The management of these wastes can be supervised by the government with its regular monitoring, because the waste disposal with its economic benefits can bring the sustainability for both environment and industrial concern [8].

2. Nature and generation of horticultural waste

The affluence of horticulture in industrial and environmental perspective is greatly significant today. Whereas there are some phenomena arising day by day with management and utilization of horticultural wastes. The nature of wastes in horticultural end is multidisciplinary. Some create chemical hazard; some are alarming for their biological and thermal point of view also. Postharvest handling and storage occur about 54% of wastes that is upstream, while 46% happens "downstream," at the processing, distribution, and consumption stages [9]. These wastes disposal is our major concern in case of sustainable waste management.

2.1. Wastes evolving during horticultural production chain

The pragmatic scenario of waste evolving is associated with the increasing of population. With higher population, increasing rate demands the higher agricultural produce. In other words, more food demand may arise with the population increasing rate.

Agricultural production nowadays is more than three times than the last five decades [10]. With technological advancement, the productivity may increase in horticultural sectors also. On the contrary with the increasing productivity, it generates the higher quantities of wastes also. Some of them are green wastes, and some are recyclable solid wastes.

2.1.1. Farming activity in horticultural production system

Mainly this sector may generate waste most in quantities. The whole process demands lots of intercultural operations such as training, pruning, thinning, earthing up, etc., of various fruits and vegetables can provide some wastes. For example, leaf residues, debris, dead leaves. However, these wastes sometimes added the additional organic matter in the soil. But if the maintenance is not sound enough in horticultural production chain, then the waste becomes burden for the environment. If we enlighten on the data (**Table 1**) given by Gmada et al. in 2019, according to their own supervision in the farm of Almeria, there are high amount of wastes distribution in various horticultural production systems. Greenhouses have the higher wastes in 39,215 ton out of 90,738 tons of total wastes, which is 43% of the total waste. Another approach of waste getting is disinfection having the second highest waste getting percentage that is 23%. So, there are different steps and period of waste getting and without waste management of horticultural products we the environment will be depleted day by day at the negative manner.

2.1.2. Chemical wastes during cultivation

These wastes are generated from the continuous use of pesticides, insecticides, and herbicides during the cultivation. These are mainly solid wastes such as pesticides containers, bottles. The activities of using these types of chemicals in developing countries are mostly handled by the rural uneducated farmers. So, the disposal of these types of solid wastes usually gets ignorance by the farmers or the users. Such types of ignorance result in the degraded mode of the environmental balance. About 2% of pesticides usually remain unused in the containers, and then the disposal of these hazardous material is done by the throwing these into the nearest ponds or on the open field condition; the ultimate environmental issues may arise by this as food poisoning, water pollution, air pollution, etc., by this type of ignorance [12].

Function	**Weight**		**Volume**	
	t	**%**	**m^3**	**%**
Greenhouses	39,215	43	49,798	27
Substrates	1219	1	1598	1
Water storage	576	1	730	0
Disinfection	21,061	23	24,066	13
Shading	10	0	10	0
Transplanting	698	1	40,714	22
Tunnels	2259	2	2429	1
Padding	4900	5	5065	3
Supporting system	6448	7	4891	3
Irrigation	4967	5	20,760	11
Plant protection	4034	4	17,333	9
Pollination	2469	3	26	0
Harvesting	2883	3	19,630	13
Total	90,738	100	187,050	100

Table 1.
Annual distribution of waste according to their function of Almeria [11].

2.1.3. Postharvest wastes

Postharvest food loss is any loss in physical weight, edibility, nutritional quality, caloric value, consumer adequacy happens between the period of reap and the time it reaches the consumer, while food waste is a subset of the food losses [13], and this might occur through human activity or inaction such as discarding produce, not consuming accessible food before its expiry date, or taking serving sizes beyond one's ability to consume [14]. Horticultural crops are highly perishable products. As it is perishable so that handling and the maintenance are really tough. For this kind of phenomena, the developing countries are the real sufferers of this type of problems. Postharvest loss and wastes of perishable commodities in horticulture are up to 60% depending on the seasons, commodity, and the region of production [15]. So, the wastes after harvesting threaten the sustainable environmental security with environmental pollution. Postharvest loss is not the issue of reduction of food availability for the consumers; it may cause negative externalities to the societies with the increasing cost effect of waste management, greenhouse gas production, and loss of scarce resources used in production [16].

2.1.4. Unconsumed waste foods and kitchen waste

One part of the world's population is struggling every day with the hunger and scarcity of food, whereas in some parts of the world, people waste food without thinking about the food security. Horticultural foods such as vegetables, fruits, and grain crops are wasted daily in our home and appear as the kitchen wastes. In

Biowaste	Bioactive compounds
Avocado peel and seed	Phenols, carotenoids
Tomato peel	Flavonols, phenolic acid, flanones, carotenoids
Banana peel	Phenols, carotenoids, flavonols, flavonoids
Mango peel and seed	Phenolic acids, flavonoids, flavonols, gallotanins, carotenoids, bioactive lipids
Pineapple by products	Phenols, cinnamic acid, amino acids, proteins
Citrus peel and seed	Flavonones
Pomegranate peel and seed	Bioactive lipids, anthocyanins, ascorbic acid
Orange peel	Phenolic acids
Watermelon peel	Anthocyanins
Apple peel	Flavonoids and anthocyanin
Papaya peel	Carotenoids, amino acids, proteins
Apple pomace	Flavonoid and anthocyanin
Carrot pomace	Carotenoids
Onion waste	Quercetin
Red beet waste	Betalins
Potato peel	Phenolic acids
Tomato peel	Lycopene

Table 2.
Bioactive compounds identified in different fruits and vegetables [19, 20].

America, horticultural wastages consist of fruits nearly 20%, vegetables 30%, others 25% [17]. General estimation of food waste annually is about trillion US dollars [18]. The whole world scenario is also alarming in this concern.

2.1.5. Industrial horticultural wastes

There are lots of food processing industries. These industries use some hazardous materials for food processing such as coloring agents, dyes, by-products such as banana peels, coconut husks, and other extraneous bioactive compounds phenols, flavonoids, flavanols, anthocyanins always evolved in the processing industries as by-products (**Table 2**). Heavy and rapid disposal is required for this kind of wastes. If it is not disposed with the time, then it will appear as a biggest threat for human survival and environmental balance. Higher emissions of pollutant make the environment more vulnerable. So, without utilizing these types of compounds it can be delectable enough to make our environment polluted.

3. Horticultural wastes as environmental concern

Environment day by day is threatened with the undisposed wastes derived from various sources of horticultural sectors, from both farming and industrial perspective. Air, water, and soil are major three components of the environment that get affected by the pollutants derived from the horticultural wastes.

3.1. Impact of waste on air quality

Sometimes, we disposed some waste with the burning. But these types of waste management are not fruitful always as burning of crop stubbles possesses some hazardous emissions of many harmful gaseous components. As a result, the atmosphere represents monoxide, nitrogen oxide, nitrogen dioxide, sulfur dioxide, methane associated with other toxic hydrocarbons. These types of dangerous gases and particulate matters make a negative impact on air and are harmful for both human and animal health (**Table 3**) [21–23].

Besides burning of crop stubbles, nitrous oxide is derived from microbial processes in cultivated soil and manures. Machineries used in crop cultivation require

Category	Pollutants[x]	Source
Particulates	SPM (PM100)	Incomplete combustion of in organic material, particle on burnt soil
	RPM (PM10) FPM (PM25)	Condensation after combustion of gases and incomplete combustion of organic matter
Gases	CO	Incomplete combustion of organic matter
	NO_2 and N_2O	Oxidation of N2 in air at high temperature
	O_3	Secondary pollutant, form due to Nitrogen Oxide and Hydrocarbon
	CH_4/Benzene PAH5	Incomplete combustion of organic matter Incomplete combustion of organic matter

[x]*SPM small particulate matter, PM particulate matter; FPM fine particulate matter.*

Table 3.
Major air pollutants emitted during crop residue burning [24].

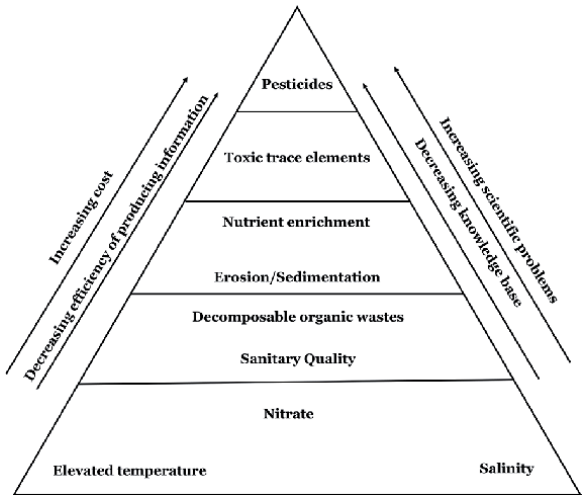

Figure 1.
Water pollution pyramid [25].

	Impacts	
Farm activities	**surface water**	**Groundwater**
Tillage/plowing	Sediment/turbidity: sediments carry phosphorus and pesticides adsorbed to sediment particles; siltation of river beds and loss of habitat, spawning ground, etc.	
Fertilizing	Runoff of nutrients, especially phosphorus, leading to eutrophication causing taste and odor in public water supply, excess algae growth leading to deoxygenation of water and fish kills.	Leaching of nitrate to groundwater; excessive levels are a threat to public health
Manure spreading	Carried out as a fertilizer activity; spreading on frozen ground results in high levels of contamination of receiving waters by pathogens, metals, phosphorus and nitrogen, leading to eutrophication and potential contamination	Contamination of groundwater, especially by nitrogen.
Pesticides	Runoff of pesticides leads to contamination of surface water and biota; dysfunction of ecological system in surface waters by loss of top predators due to growth inhibition and reproductive failure; public health impacts from eating contaminated fish. Pesticides are carried as dust by wind over very long distances and contaminate aquatic systems thousands of miles away (e.g. tropical/subtropical pesticides found in Arctic mammals).	Some pesticides may leach into groundwater causing human health problems from contaminated wells.
Feedlots/animal corrals	Contamination of surface water with many pathogens (bacteria, viruses, etc.) leading to chronic public health problems. Also, contamination by metals contained in urine and feces.	Potential leaching of nitrogen, metals, etc. to groundwater
Irrigation	Runoff of salts leading to salinization of surface waters; runoff of fertilizers and pesticides to surface waters with ecological damage, bioaccumulation in edible fish species, etc. High levels of trace elements such as selenium can occur with serious ecological damage and potential human health impacts	Enrichment of groundwater with salts, nutrients (especially nitrate).
Clear cutting	Erosion of land, leading to high levels of turbidity in rivers, siltation of bottom habitat, etc. Disruption and change of hydrologic regime, often with loss of perennial streams; causes public health problems due to loss of potable water.	Erosion of land, leading to high levels of turbidity in rivers, siltation of bottom habitat, etc. Disruption and change of hydrologic regime, often with loss of perennial streams; causes public health problems due to loss of potable water

Table 4.
Wastes effect in different horticultural operation on water pollution [27].

fuel combustion resulting in the rapid production of CO_2. The ultimate result of air pollution leads to the temperature rising, ecological disbalance, and degradable sustainability of the environment.

3.2. Water contamination through horticultural pollutants

Only the industrial solid wastes with heavy metals are not the headache for water contamination today. Agricultural wastes, more specifically the horticultural wastes within horticultural cultivation system and processing by-products, can hamper the water quality in various ways. Fertilizer and other pesticide chemicals are responsible for both ground and surface water contamination. Toxic trace elements make the essential nutrients unavailable, and beneficial soil-borne microorganisms become extinct. Water pollution pyramid (**Figure 1**) stated that groundwater use has become unsafe for the toxicity of the chemicals. Erosion, sedimentation, salinity are the typical after-effects of continuous cultivation system.

Only agricultural field makes the water polluted about 70% worldwide [26]. Besides, different intercultural operations have the major influence in water contamination (**Table 4**).

3.3. Impact on soil

Long-term fertilization and indecomposable plastics solid waste make the soil barren for the crop cultivation. Besides, some plant residues contain the toxic chemicals (secondary metabolites, volatile terpenes, phenolic compounds), which can suppress the growth and production of other crops. This type of phenomenon is addressed as crop-crop allelopathy. Postharvest residues are mainly the source of this kind of allelopathic effects [28].

4. Technique of horticultural waste management

Horticultural waste such as the peels, seeds, and other constituents of vegetables and fruits that contain high amount of phytochemical compounds and essential nutrients are used to produce different industrial products. It can be utilized to extract as well as obtain bioactive compounds that can be used in food, textile, and pharmaceutical industries as shown in **Figure 2**.

The techniques of horticultural wastes management consist of different applied strategies for different kinds of wastes. Generally chemical, biological, biofuels, and thermal strategies are followed throughout the world (**Table 5**).

4.1. Bioactive compound of agricultural waste

4.1.1. Pectin, starch, cellulose as biopolymers

From various by-products we find some starch, cellulose; where starch is a white granular, organic compound with soft, tasteless powdery appearance insoluble in cold water, alcohol or different solvents, and cellulose found by peeling of horticultural crops as it is available in primary cell wall of green plants [30] Amylose and amylopectin are the branched form of starch, whereas linear polymer is the simplest one [31] Starch nowadays is produced from banana peels, corn, pea, potato, cassava roots. Banana peels can be processed for bioplastic production and sometimes sodium metabisulfite used as antimicrobial agent, glycerol used for more flexibility. Degradation of bioplastics produced from starch starts after 3–4 months

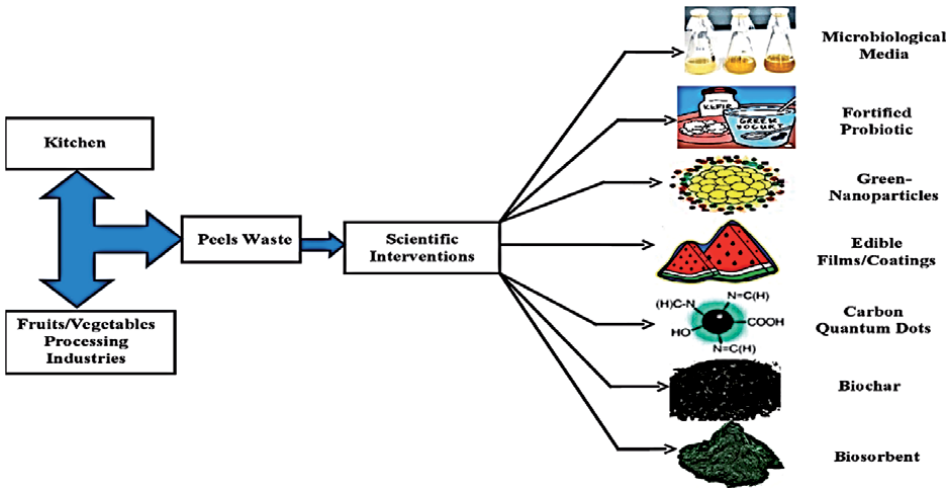

Figure 2.
Utilization of fruits and vegetable peel-based waste into novel industrial products [29].

Chemical	Biological	Biofuels	Thermal
• Starch, pectins, cellulose • Natural colorants Dietary nutrients or fiber • Bioactive compounds	• Animal feeding • Composting • Vermiculture • Substrate for microbial growth	• Bioethanol • Biogas	• Incineration • Pyrolysis

Table 5.
Strategies for horticultural wastes management [19].

date of production [32] By this, starch-producing strategies can give the commercial aspects for many emerging entrepreneurships. This is also nonhazardous for the environment because it is readily disposable after a short period of time. Not only banana but also cassava will completely be degraded on the ninth day after production of bioplastic [33] On the contrary, plastic materials are nondegradable products that can hamper the balance of the environment. Cellulose sometimes coverts into starch or glucose by decomposing called cellulolysis with the help of microbes such as *Trichoderma reesei* and *Aspergillus terreus*. Then it is used as a material for bioplastic production (**Table 6**) [34].

4.1.2. Utilization of wastes as coloring agent, dietary fiber, and prebiotic compounds

4.2. Biological approaches for wastes mitigation

4.2.1. Animal feed

Animal feed can be the good approach for productive waste disposal. That can provide manures, which influence reduction of synthetic fertilizer use tendency. Fermentation industries are established on the basis of wastes types (**Table 7**). This provides the ultimate economic outcome without creating the environment hazards in nature.

Food product	By-product	Formulation/Storage conditions	Dietary fiber/prebiotic compound	Optimal dosage (s)	Impact on senzorial characteristics	Other impacts
Cake	Potato peels	Powder (drying- > grinding)	Dietary fiber	5%	No major changes in the product were noticed, just more darkness color.	Increasing the strength and elasticity of the dough.
Donut	Carrot pomace	Powder (drying- > grinding- > sift)	Dietary fiber: pectin, lignin, cellulose, hemicellulose	6.45%	The sample showed a smaller volume. Consumers have suggested adding a glaze.	Significant impairment of physico- chemical properties.
Biscuits	Carrot pomace	Powder (whitening-grinding-sif)	Dietary fiber: pectin, lignin, cellulose, hemicellulose	10%	—	Neutralization of free radicals
Eriste (Turkish noodle)	Grapes, pomegranates, rosehips seeds	Powder (grinding- > sift)	Dietary fiber	10%	The sample enriched with pomegranate seed powder obtained the highest appreciations from a sensory point of view.	Increase in antioxidant activity.
Corn chips	Mango peels	Powder (freeze drying)	Dietary fiber	10–15%	Improving and maintaining the smell, texture, color and aroma.	Increasing the content of total phenolic compounds.
Ice cream	Red pitaya peels	Powder (grinding- > sift)	Dietary fiber: pectin, lignin, cellulose, hemicellulose	1%	Melting rate and color were not affected.	Improving rheological qualities and increasing nutritional value.
Ice cream	Grapefruit peels	Stem-shaped crystals	Nanofibril cellulose	0.4%	Texture improvement.	Reducing caloric intake.
Agitated type yogurt	Carrot pomace	Powder	Dietary fiber: pectin, lignin, cellulose, hemicellulose	1%	The color and smell of the sample were affected and strawberry flavor was added to improve them.	Reducing syneresis.
Chocolate	Grapes pomace	Powder (drying- > grinding- > sift)	Dietary fiber and prebiotic compound: lignin, cellulose, oligosaccha- ride	3–5%	At a higher dosage there is a slightly bitter taste due to phenols. The greatest impact on the product occurred in the particle size.	Water activity and stability increased.

Food product	By-product	Formulation/Storage conditions	Dietary fiber/prebiotic compound	Optimal dosage (s)	Impact on senzorial characteristics	Other impacts
Instant drinks	Mango peels	Powder (bleaching- > drying with hot air)	Prebiotic compound	5 g/250 mL	During storage, the sensory characteristics decrease.	Improvement of phyto- chemical parameters and stability increases during storage.
Vienna sausages	Pineapple pomace	Powder (pressure steaming– + lyophilized or hot air dried)	Dietary fiber: lignin, cellulose, hemicellulose			The educing effect on nitrites, moisture, shear strength and shrinkage was obtained in sausages, while carotenoids and antioxidant polyphenols increased. Increased
Buffalo meat	Apple pomace	Powder	Dietary fiber: lignin, cellulose, hemicellulose	6%	The firmness increased, and the color became redder and darker.	Cooking efficiency, water retention capacity, pasta diameter.
Flour	Feijoa peels	Steam discoloration-rice bath- > drying in a convective oven- > grinding	Dietary fiber: lignin, cellulose, hemicellulose	—	—	Alternative source of bioactive ingredients.
Powder	Olive pomace	Liquid-enriched pomace powder (the liquid fraction was lyophilized and the solid fraction was dried)	Dietary fiber: pectin, lignin, cellulose, hemicellulose		—	Food preservative and source of mannitol.

Table 6.
Recent (last 5 years) report of utilization of fruits and vegetable wastes, dietary fibers, and prebiotic compounds in different food products [35].

Plant origin	Fermentation industry
Bran	Grain
Waste flour	Sugarcane industry (molasses, bagasse)
Wastes from grain-cleaning process	Potato distillers soluble
Wheat	Brewery waste
Corn	Bacteria and fungi biomass
Rye germs	Winemaking industry (grape pomace)
By-products of oil industry	Citrus by-products (molasses, citrus-activated sludge)
By-products of sugar and starch industry	Anthocyanins
By-products of fruit and vegetable industry	Effluents from biogas production
Plant by-products (husk and pods)	Dairy industry

Table 7.
Horticultural wastes were used in animal feed [19].

4.2.2. Composting for waste disposal

Compost is most demandable nutrient source in the crop field. This approach allows growers to spend less money for their initial cultivation inputs. Also, higher yield will be observed by using compost instead of synthetic fertilizer [36]. Different types of composting methods are used on the basis of grower's choice and wastes types (**Figure 3**).

4.2.3. Biofuels (bioethanol, biogas)

Waste can be disposed through converting the wastes and by-products into biofuels. Bioethanol and biogas production nowadays appears as the most sustainable waste management program, which has some significant economic values.

Bioethanol can be processed through horticultural by-products such as carrot peels, banana peels, and other crops parts, which previously can be dried in the sun. The product can be ground and sieved for further processing. After that, the products can be pretreated with 1 N NaOH for 2 hours. Then draining or in other words alkali subsequent washing can be done. Enzymatic hydrolysis by cellulase enzyme leads to saccharification. Then the inoculation of *Saccharomyces cerevisiae* is required. Then the fermentation and distillation are done for bioethanol production (**Figure 4**).

The other way for wastes disposal as the biofuel source is biogas production. Nowadays we can see the rapid adaptation of this disposal system in our rural areas also.

In case of biofuel products, horticultural by-products can be utilized in effective way. Different content of organic matters can yield the sufficient amount of methane gas (**Table 8**).

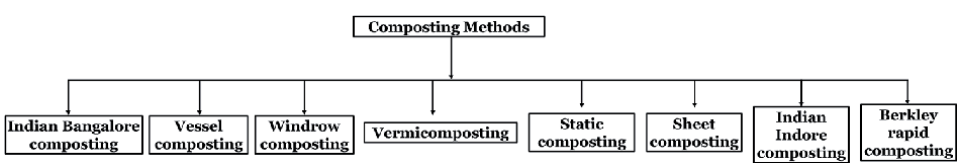

Figure 3.
Different composting methods [37].

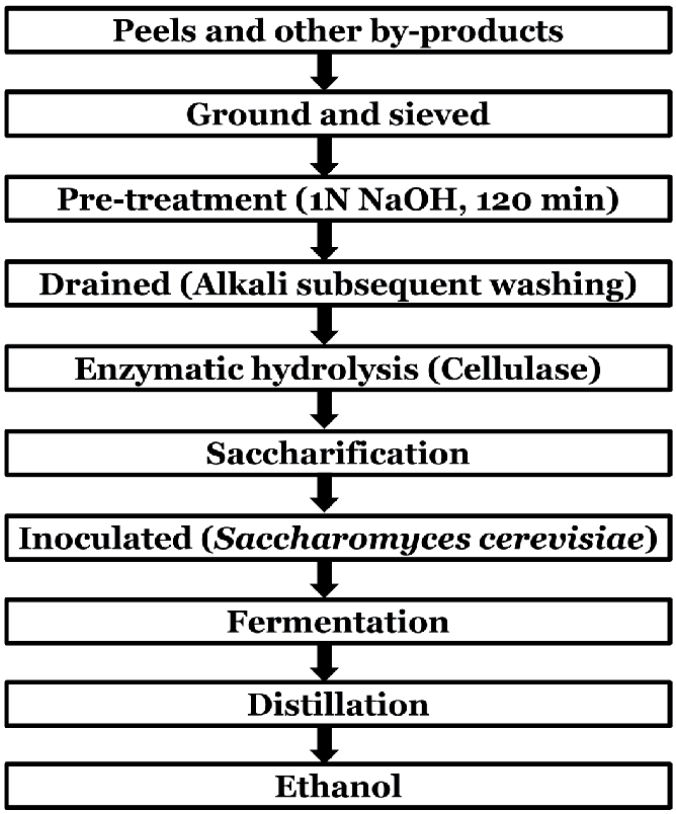

Figure 4.
Flow chart for bioethanol production by cellulase enzyme [38].

Substrate	Organic dry matter in %	Methane yield in Nm^3/t ODM
Banana peel	87–94	243,322
Citrus waste	89–97	433,732
Coriander waste	80–86	283,325
Mango peel	89–98	370–523
Oil palm fiber	94	183
EFB	79–84	200–400
Onion peels	88	400
Pine apple waste	93–95	355,357
Pomegranate	87–97	312–430
Sapot peels	96	244
Tomato waste	93–98	211–384
Water hyacinth	81	211–310
Coffee waste (pulp)		380 (biogas yield)

Table 8.
Biogas generation rate from the horticultural by-products [39].

5. Negative consequences of traditional disposal operations

5.1. Incineration

Combustion of wastes materials to achieve waste to energy is called incineration. High-temperature thermal treatment converts wastes into ash, flue gas, and heat. It requires localized combined heat and power facilities to encourage its heating process. Japan, Denmark, Singapore, and Netherlands follow this technique usually to dispose the wastes [40]. This method can reduce wastes up to 90%; but this is one type of waste reduction process rather than the disposal process as it is associated with the fire disaster and production of greenhouse gases [41]. Energy produced as coal could save about 2.26 MT of CO2 eq/year [42].

5.2. Pyrolysis

Waste composition can determine the effectiveness of pyrolysis. It has several advantages comparing the incineration process. Lower temperature is preferable, and the plant for pyrolysis more flexible enough; product derived from pyrolysis can be converted through alternating the temperature and heating performance [43]. The pollutant emissions are lower in this disposal process as there is absence of oxygen and with low processing temperature, although emissions of other compounds simultaneously could increase with lower oxygen ratio [44]. Biochar production can be done by fruits and vegetable peels and other residues from, for example, spinach, bananas, peas, and tomatoes [45]. There are two types of pyrolysis, i.e., slow and fast. For biochar and gas production, slow pyrolysis is preferable, and bio-oil can be produced better in fast pyrolysis (**Figure 5**).

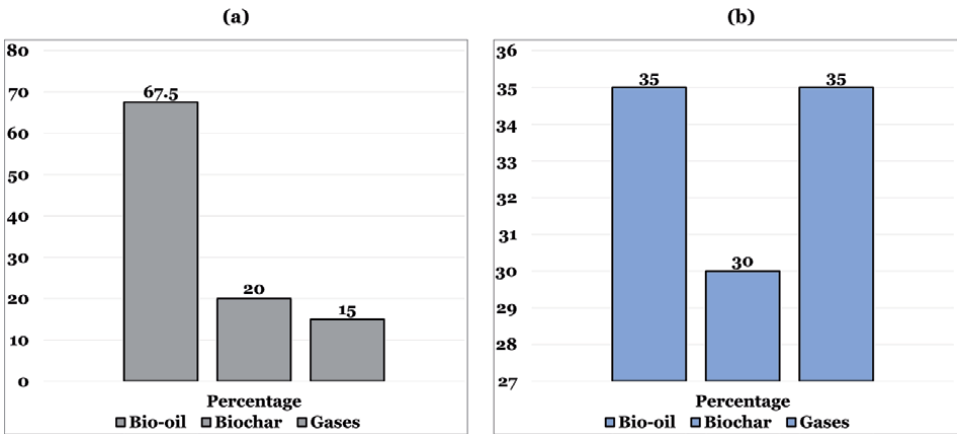

Figure 5.
Bar diagram of pyrolysis produced product. (a) Fast pyrolysis and (b) slow pyrolysis [46–47].

6. Prospects of horticultural waste management

It is obvious that waste disposal is not an easy task as it requires bigger margin of resources and right methods to minimize its after-effects. All the techniques or methods of repealing wastes are not efficient enough always. Food recovery hierarchy published by US EPA showed that there are different methods or approaches are

proficient at different level. Landfilling and incineration (combustion of the waste materials) are the last resort of wastes disposal, because sometimes it is harmful for our environment. Soil pollution and abundance of toxic gases are visible by this kind of disposal system. Then the composting creates a nutrient-rich soil amendment. It requires specialized area away from the home, and it requires more time for disposal. Industrial uses of wastes are just above from the composting in that pyramid as it provides waste oils for rendering and duel conversion and food scraps for digestion to recover energy. Lots of commercial industries are developing today with the new hope with horticultural by-product establishing. Although some wastes also are considered developing the industries but commercial exposure may be spread rapidly. Main effective approach will be the source reduction and sometimes we waste food more than we consume so that extra food can be donated to food banks, shelters can reduce the possibility food wasting. Public awareness is the big thing for food waste management in horticultural sectors also (**Figure 6**).

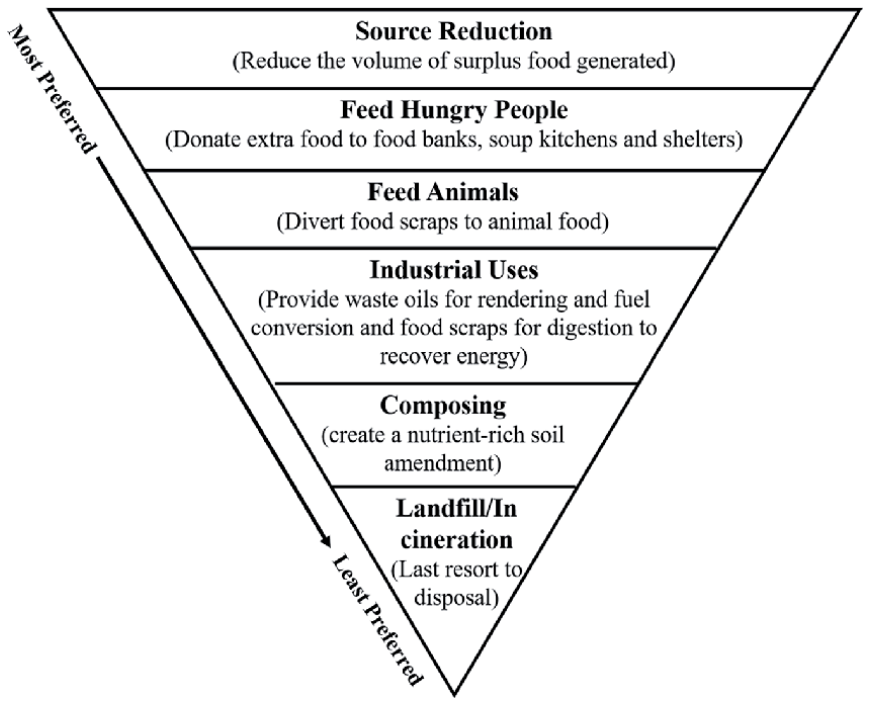

Figure 6.
Food recovery or management hierarchy [48].

7. Conclusion

Various environmental concerns and some economic benefits demand the appropriate disposal of horticultural wastes. Minimizing of wastes can maximize the environmental stability. However, people are not so aware about the impact of horticultural wastes. So, the proper awareness with the effective implementation of wastes is a crying need for today. Meanwhile, sustainability can be brought through adapting the modern disposal methods with longer effects and economic flexibility. In addition, the growth of the wastes disposal industries also gives the new dimension for the sustainable waste management. Finally, it can be enunciated that waste management provides green ecology, which can serve environmental stability with industrial prosperity.

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Section 2

Pectin Biotechnology

Chapter 3

Biotechnology Applications in the Pectin Industry

Asra Hamidi (Ataran)

Abstract

Pectin and/or pectin-like polysaccharide (PLP) is one of polysaccharides contained in the plants and algae cell walls, That's known as a polymer branched from galacturonic acids. Pectins are widely used in industry to remove heavy elements, gel and stabilize materials. Furthermore, its antioxidant properties are considered medically and in healthy eating policies. "Pectin is composed of D-galacturonic acid linked by α-1, 4-glycosidic linkage and in most cases, pectins are classified according to their degree of esterification (DE), which represents the ratio of galacturonic acid groups esterified in the structure of the pectin polysaccharide. The high methyl (HM) ester is a polymer that is methyl esterified in more than 50% of its carboxylate monomers, and conversely, the low methyl (LM) ester is a pectin with a degree of esterification of less than 50%. The bioactive properties of pectin polymers are very wide. For example, pectins, with their antioxidant properties, are anti-cancer and anti-tumor, and help heal patients undergoing chemotherapy. Pectin polymers can help improve diabetes and lower cholesterol. In addition, pectin has received much attention in medicine due to the importance of hydrogels, nanofiber mats and nanoparticles." The purpose of this chapter is to review and introduce possible applications of biotechnology in pectin industries. We review sections on agricultural production and the enzymatic extraction method, as well as enzymatic-ultrasonic extraction. Finally, some suggestions are made for factory effluents and solid waste.

Keywords: Pectin, Extraction, Biotechnology, Enzyme-ultrasonic, Enzymatic, Ultrasounds, Pectin-like, Algae, marine

1. Introduction

"In general, pectin is extracted from agricultural products, especially apples, oranges, carrots, etc" [1–5]. But some environmental controls in plant breeding, as well as genetic engineering may increase the production of pectin in the plant. You may also be able to specialize more economically organisms to produce pectin.

For example, "Ajaya K Biswal, et al reported in 2015 that "Mutation of GAUT12 in *Populus deltoides* by RNA silencing results in reduced recalcitrance, increased growth and reduced xylan and pectin in a plant biofuel feedstock". The family name GAUT was coined after the discovery of Arabidopsis galacturonosyltransferase 1 (GAUT1). GAUT1 is a pectin biosynthetic homogalacturonan (HG): α-1,4-galacturonosyltransferase (GalAT) that functions in an HG:GalAT protein complex with GAUT7. The highest amount of Arabidopsis GAUT12 / irx8 was measured in cell tissues containing secondary wall that Its molecular structure is about 61% similar to that of GAUT1 monomers" [1].

"GAUT12 is thought to be a type II membrane protein and a Golgi target. If irx8 / GAUT12 mutates, GX decreases. In the Biswal study, microsomes from irx8 mutant stems didn't show any reduction in xylan XylT activity or xylan GlcAT (glucuronosyltransferase) activity compared to microsomes from wild type (WT)" [1].

Therefore, it's possible to increase pectin production in plants by UP-regulation or GAUT cloning. For any case, it is important to do a thorough study of pectin production control genes first. You may be able to DNA editing and programming for cells with greater, and more cost-effective reproduction.

Also, the environmental conditions' control of the plant, such as light, moisture, soil elements, fertilizers, temperature and etc., may affect of pectin production. Since these plants are primarily edible, this works can hurt food and agricultural policies. For this reason, in the pectin industries are primarily used, agricultural wastes.

"1.3 billion tons of human's food produced consumption is lost or wasted each year. In the meantime, 45% of food wasted are vegetative and herbal." "Food industries based on fruits and plants produce significant amounts of solid waste that can be used for animal feed, fertilizer, biochar production [6] or biogas [7]". However, the bulk of this waste is transported to landfills. While waste includes "avoidable" and "unavoidable" items; Waste of food, such as discarding edible parts of fruits that aren't suitable for the food industry, as well as other untreated/unrecycled waste, causes the spread of greenhouse gases, global warming and "carbon footprints". For this reason, the potential use of agricultural by-products and solid waste of the food industry creates a comprehensive opportunity for the development of functional ingredients industries [3].

"Pectin methyl esterases (PMEs) are important for the regeneration of pectins during plant growth. These enzymes have been reported in both plants and plant pathogens (such as bacteria and fungi). Plant and microbial PMEs aren't the same in enzyme substrate properties and pH tolerance. Furthermore, differences have also been reported between plant PMEs. Different types of PMEs act differently in response to different chemical compounds. For example, the inhibitory composition of plant PMEs, proposed by Melanie L'Enfant Jean et al, had completely no effect on the PMEs of pathogenic fungi. However, this study could open the way to control the properties of pectin methyl esterases" [2].

2. Pectin-like carbohydrates in algae

Today, algae is considered as one of the best and most cost-effective sources for many organic products. The ability to produce a product per unit time for algae can be much higher than plants. They do not need agricultural land and consume less water and resources. On the other hand, the harvest of algae in a photobioreactor can be about 24 times per year. Studies show that "the ability to produce lipids in algae is higher than plants. The percentage of oil and the quality of essential fatty acids - such as omega-3 and omega-6 - in algae is even higher than in fish oil. For these reasons, the consumption of algae - as human food - has attracted the attention of many in the international community, especially vegetarians. Also, the special properties of algae have made them the main candidates for providing new biofuel production resources" [8]. The algae ability of produce is not limited to lipids, some types of algae produce the high of proteins, carbohydrates, etc. Also with the use genetic engineering or even controls of the culture medium, they can easily be made more specialized.

The use of algae, to provide sources of pectin extraction is very attractive; Because the use of algae as a source, does not require agricultural land, abundant

water and abundant and expensive resources, also its harvest per unit area is faster and more. On the other hand, increasing the percentage of pectin production in plants can be contrary to global food supply and biofuels policies.

"Magdalena Eder & Ursula Lütz-Meindl, identified pectin-like carbohydrate molecules in the green alga *Netrium digitus*. Pectins known to be involved in cell-wall expansion and representing major components of mucilage were the main objectives of this study. In more evolved plants, low methyl esterified pectins occur at cell corners, in middle lamellae and around air spaces, It's thought that the placement of pectins in these areas was done with the aim of creating resistance to environmental stresses. By forming a stable gel using calciumBridges, low methyl-esterified pectins prevent separation of cells as frequently induced by stress factors" [9].

"Lee, Kyung-Ah et al also reported that they were able to extract pectin-like polysaccharides from marine algae. They extracted Pectin-like from 5 kinds of microalgae and 9 kinds of macroalgae with different extraction methodologies. High yield of PLP was extracted in distilled water (DW) as 21.06±3.5% from *A. maxima*. In general, pectin-like extraction from macroalgae was more satisfactory than microalgae. In acidic condition (AC), PLP from Undaria pinnatifida was not precipitated. However, the yields of galacturonic acid was higher in Hizikia fusiformis (80.28±4.58%) and Laminaria japonica (65.85±0.61%), respectively. In biological activity tests, Fe2+ chelating activity was 26% higher than pectin from citrus peel as control. ABTS scavenging assay showed 100% antioxidative activity based on DW extraction from Ecklonia cava, whereas not detected from other macroalgae. The results of this study show new hopes for the use of gelling and stabilizing properties of PLP in various industries" [10].

"The total amount of carbohydrates in macroalgae is about 40 to 65% and in microalgae is about 10 to 50%. Kyoung-Ah Lee et al reported that they investigated the composition of PT and the anti-oxidant activities of 5 species of micro-algae (Spirulina maxima, Leptolyngbya sp, Tetraselmis sp, Dunaliella sp, and Chlorella sp) and 9 species of macro-algae (Saccharina japonica, Sargassum fulvellum, Undaria pinnatifida, Ecklonia cava, *Gracilaria verrucosa*, *Gelidium amansii*, Sargassum fusiforme, *Ulva pertusa*, and Sargassum horneri). Furthermore, Edirisinghe, S.L. et al and Chandrarathna, H.P.S.U. et al observed immunologic values of Spirulina maxima PT that have the potential to modulate gut microbial population, en- hance the expression of immune related genes, and boost gut morphology in zebrafish larvae" [10, 11].

"Rajapaksha, Dinusha C. et al and Edirisinghe, S.L. et al reported that the performance of pectin-like extracted from Spirulina.maxima was significant for wound healing. Also, D.S. Domozych et al wrote that the green alga Penium margitaraceum shows pectin metabolism in its cell wall. In addition, EDER et al extracted Pectin-like polymers from the cell wall and mucosa of the green alga *Netrium digitus*. In fact, many researchers have been able to extract pectin-like molecules from algae and have studied the properties of these molecules; But we still need more evidence on the effectiveness and industrial value of pectin-like extraction" [11].

"Kyoung-Ah Lee, et al reported, that they were able to extract pectin-like biomolecules from 5 species of microalgae and 9 species of macroalgae. MP extraction yields were higher in the distilled water (DW) extracts of Spirulina maxima and *Ulva pertusa* (yield extractions of 21.90±1.12% and 18.80± 0.97%, respectively). These results confirm that a large amount of MP was extracted by DW from marine algae, and MP is unlike the pectin derived from land plants. The MP extraction conditions were established using different solvents for each marine algae, and optimum extraction conditions exist for each species. Regarding biological activity, The MPs from Ecklonia cava and Sargassum horneri showed 99% ABTS radical scavenging activity, and the Fe2+Chelating activity of the MP from Dunaliella sp. Was confirmed to be higher than those of other MPs. The results of this study potentially

indicate the potential for industrialization of pectin-like extraction from algae. This article also shows that the development of the use of DW materials in industry can help reduce environmental pollution" [10].

3. Pectin extraction and biotechnology

"Approximately 30% of the primary cell wall in apples and citrus fruits contains PT pectin. There are several methods for extracting PT; Such as extraction with acid, hot water, enzyme, microwave, ultrasonic as well as combined methods. Acid and hot water extraction are the oldest methods in the pectin industry. The biggest drawback of traditional methods is the degradation of pectin polysaccharides and the long extraction time. In contrast, enzymatic extraction has many advantages such as no degradation of pectin polymers, low extraction temperature, shorter extraction time, reduction of environmental pollution, requires very low acidity and so on. For this reason, various green extraction techniques have received much attention" [11].

- biotechnology methods in pectin extraction include: enzymatic extraction and enzymatic-ultrasonic extraction (and etc). Which we examine.

4. Enzymatic extraction of pectin

"The analysis in **Table 1** shows that, the polysaccharides xylan and xyloglucan form the crosslinker between pectin, cellulose and hemicellulose. For this reason, the use of cellulase and xylanase can break the bond between these polysaccharides and separate pectin molecules from the cell wall. Based on studies and laboratory evidence, the highest enzymatic extraction efficiency of pectin is in the simultaneous use of cellulase and xylanase. Because using a mixture of cellulase and xylanase, it destroys the bond between xylan, xyloglucan and cellulose and causes the separation of pectin. While enzymatic treatment with xylanase alone reduces the extraction efficiency. The reason for this reduction in efficiency is probably the strong properties of the bonds between hemicellulose xyloses, not the bonds between hemicellulose and pectin or cellulose. The use of xylanase can improve cellulase function by degrading xylan / xyloglucan from the SBP matrix and further release pectin. However, the use of a higher cellulase ratio (2:1) reduced the pectin extraction efficiency in the study by Abou Elseoud et al. However, this may be due to pectinase activity remaining in the cellulase enzyme derived from Trichoderma Iongibrachiatum – reported by him. Because pectinase enzymes cause the hydrolysis of pectin polymers to soluble sugars, thereby reducing the efficiency of pectin extraction.

Comparison of pectin extracted in the best conditions of traditional extraction with sulfuric acid (temperature 85°C, pH = 1, time: 2 h) against pectin extracted by combined treatment of xylanase and cellulase enzymes (8.28%) shows that the extraction efficiency is similarity with the acid extraction method (5.26%). In addition, the maximum enzymatic extraction efficiency of pectin in the Abou Elseoud study is equal to or greater than the acidic extraction of beet pulp" [4].

In enzymatic Pectin extraction GalA efficiency from passion fruit peel were between 17.0 and 25.8 g/100 g of dry peel, which were similar to those obtained for the more commonly used citrus peel substrates with PPase-SE. Efficiencies were also comparable with those obtained from lemon pomace using a different polygalacturonase from Aspergillus niger and from pumpkin using a cellulase from Trichoderma viride and a multi-enzyme crude extract from *Bacillus polymyxa*. GalA efficiency from acid extraction was 15.9 ± 0.1 g/100 g dry peel, Which shows a much lower result

Fruit/fruit by-product	Yield of pectin	Treatment conditions			pH	Galacturonic acid	Degree of esterification
		Temperature (°C)	Time (min)	Acid			
Mango peel	5.4%[a]	85	30	Nitric acid	2	80.71%	67%
Pomelo peels	3.11%[b]	90	90	Hydrochloric acid	2	NS	NS
Cubiu fruits peels	14.2%[c]	Temperature not specified (boiling)	120	Nitric acid	1.5	72%	62.0%
Cubiu fruits peels	9.6%[c]	100	120	NS	NS	79%	56.9%
Ponkan peels	25.6%[a]	Temperature not specified (boiling)	100	Nitric acid	1.6	84.5%	85.7%
Cornelian cherry fruit whole	0.83%[a]	Room temperature	60	NS	3	59.1%	84%
Pomegranate peel	8.5%[a]	86	80	Nitric acid	1.7	62%	75%
Honey pomelo peels	17.5%[a]	85	80	Hydrochloric acid	1.24	749 g/kg	76.6%
Durian rinds	9.1%[a]	86	43	Hydrochloric acid	2.8		
Fresh watermelon rinds	19.3%	Temperature not specified (boiling)	60	Nitric acid	NS	74.2%	63%
Pomegranate peel	11.34%[c]	88	120	Citric acid	2.5	80.95 g/100 g	53.09%
Wolf apple unripe fruit pulp	33.68%[a]	80	30	Nitric acid	1	NS	77.15%
Main harvested kiwi fruit	3.27%[a]	50	60	Citric acid	2.8	56.08%	82%
Early harvested kiwi fruits	1.43%[a]	50	60	Citric acid	2.8	48.80%	88%
Main harvested kiwi fruit	3.27%[a]	50	60	Water	NA	51.87	84%
Early harvested kiwi fruits	1.01%[a]	50	60	Water	NA	42.88	90%
Citron peel	21.85%[a]	90	180	Water	NA	NS	77%

[3], p. 6.
a Considered on dry weight of example [dry weight of extracted pectin [g] ÷dry weight of example [g] ×100]
b Considered on dry weight of oil free example [dry weight of extracted pectin [g] ÷dry weight of oil free example [g] ×100]
c Considered on dry weight of alcohol insoluble residue of example [dry weight of extracted pectin [g] ÷dry weight alcohol insoluble residue [g] ×100];
NA: not applicable / NS: not specified

Table 1.
Repercussion of classical extraction method on the efficiency, quality, degree of esterification and galacturonic acid amount of extracted pectin from tropical and sub tropical fruit /by products.

than other extraction conditions ($p < 0.05$). This was probably due to the decomposition of a percentage of soluble GalAs due to very low pH and high temperatures. Therefore, due to the lower extraction temperature as well as the milder pH required for enzymatic extraction; The performance and quality of pectin molecules obtained by enzymatic extraction method have been reported to be significantly higher than the chemical method. A significant number of reports show similar results in studies of pectin from passion fruit. However, most published reports are about the extraction of pectin from the dried peel. Juliana Vasco-Correa and Arley D. Zapata-Zapata [5], used the fresh peel with a relative high particle size, which it need to less energy intensive since high energy would be needed for drying and milling the peel. In Liew et al. study and Kulkarni & Vijayanand obtained maximum efficiency of 14.6 g/100 g of peel and 14.8 g/100 g of peel, respectively, by extracting pectin from passion fruit peel citric acid at pH 2 was used, which are similar to the conditions and results of the chemical extraction performed in the Vasco-Correa's studys. Canteri et al. obtained a slightly higher yield of 20.3 g/100 g of rid flour from passion fruit, using nitric acid for the extraction. Contreras234 Esquivel et al. achieved a yield of 25 g/100 g of dry passion fruit fiber using citric acid and autoclaving for 20 min. Kliemann et al. (2009) Enzyme loading had a significant effect on GalA yield ($p < 0.05$) (**Table 1**) [5].

The results do not show a significant difference between the performance of GalA prepared at 30 and 40 U / mL. But these two groups recorded higher solubility than 20 U / mL. Therefore, increasing the enzyme charge to 30 U / mL can improve the solubility of GalA, but increasing the enzyme load can not produce more positive results. According to the results, the maximum adsorption of PPase on protopectin (the pure substrate) obtained at 30 U/mL of PGase. So, 30 U/mL can considered as optimum enzyme loading. So, excessive increase in enzyme concentration at 40 U/ml not only increases project costs, but can also degrade soluble pectins, as PPase has significant endopolygalacturonase activity and can degrade internal bonds between GalAs. Although agitation speed has had a significant impact on other PPase-SE processes, in this case, it has had very little effect on GalA performance ($p > 0.05$). This is due to the excellent permeability and solubility of the enzyme anywhere, at 120–180 rpm. It's also possible that in the Vasco-Correa report, vortex flasks led to better mass transfer. Other reports indicate that agitation speeds do not have a significant effect on enzymatic heterogeneous processes in low solids loading, which in the study was about 2.5 g/100 ml. In addition, temperature and pH variables affect the rate of GalA dissolution ($p > 0.05$). Maximum yield was measured at 37° C and pH 3.0. This temperature was also the optimum value found for lemon peel pectin extraction using PPase-SE. Minimum yields were obtained at 44°C, perhaps because the enzyme wasn't stable at this heat temperature. While, changing the pH in the range of 4.0–5.0 does not cause much change; GalA performance increased significantly at pH 3.0 ($p < 0.05$). However, these results contradict the results of the study of pectin extraction with PPase-SE from lemon peel, under similar conditions and higher pH. Therefore, it is necessary to determine the best pH for each system, based on the study of the specific enzyme and substrate of the project. In the acidic extraction method of pectin, the more acidic degree of pH will be in favor of optimizing the extraction process, while the same can cause the destruction of pectin molecules. Acid also kills microbial pathogens.

The report of the periodic study of enzymatic extraction in flasks shows the maximum extraction efficiency of 21.9 g / 100 g of dry peel in 120 minutes. The longer the extraction time, the lower the efficiency and quality of the product, because there is a possibility of unwanted enzymatic hydrolysis due to the endopolygalacturonase activity of the enzyme. While some studies have used enzymatic therapy for longer than 12 to 20 hours, no significant increase in efficiency, quality, or optimization that benefits the pectin extraction industry has been reported.

Generally, yield is normally requested in scale-up of biocatalytic processes, and as the size of the stirred-tank increases, an increment of the mixing time is expected. Agitation speed in the range studied in the bioreactor had a great effect on GalA. The lower the energy consumption of a biochemical process, the greater the potential for industry expansion. This is one of the most important things to consider when planning your business. However, calculating the actual energy consumption for a floating particle heterogeneous system can be difficult without direct measurements" [5].

5. Quality of pectin molecules from enzymatic extraction

"Studies show that the highest efficiency and quality of pectin extraction is obtained by enzymatic method with simultaneous use of cellulase and xylanase enzymes. For more information, see the following data: All spectra showed the characteristics peaks for pectic rich polysaccharide as follows: stretching vibration of OH groups of carboxylic acid and alcohol at ~3432 cm − 1, stretching vibration ofC-H groups of methyl, methylene groups at ~2900 and 2925 cm − 1, stretching vibration of CO of ester groups at ~1744 cm − 1, symmetric and asymmetric stretching vibration of CO of carboxylate groups at ~1621 cm − 1 and 1433 cm − 1, amide groups of protein linked to pectin at ~1544 cm − 1, stretching vibration of C-N groups (present in protein) and also C-O bending at 1270, 1100 & 1026 cm − 1 for the C-O bonds in glycosidic linkage and alcoholic OH groups of sugars. Quantification the ratio of the intensities of carboxylic group at 1632 cm − 1 or ester group at 1744 cm − 1 to that of methylene C-H group of the backbone at 2925 cm − 1 showed that A1632/A2925 ratios were 1.49, 1.63, and 1.50 for pectin sample extracted using 1:1, 1:1.5, and 1:2 xylanase to cellulase enzymes, respectively, while A1744/A2925 ratios were 0.88, 1.0, and 0.97 for the same samples, respectively.

The results showed noticeably higher ratios of A1632/A2925 and A1744/A2925 in case of using 1:1.5 xylanase to cellulase ratio. The study of sugars in **Table 2** shows the highest galacturonic acid content in this sample. It is also shown that the highest extraction performance is obtained at this enzymatic ratio. Ester or carboxylic acid groups are another important factor in the emulsification properties of pectin" [4].

"**Table 2** shows galacturonic acid, neutral sugars, protein, ferulic acid contents, degree of esterification, and molecular weight of the extracted pectin samples using different xylanase to cellulase enzymes doses. Examination of the table shows that increasing the ratio of xylanase to cellulase from 1: 1 to 1.5: 1 in the extraction solution does not cause significant changes in the galacturonic acid content of the resulting pectins. While increasing the concentration of cellulase enzyme in a ratio of 2: 1, reduces the amount of galacturonic acid in pectin polymers. Analyzes show that the highest content of neutral sugars is related to glucose and the concentrations of rhamnose, galactose, xylose and arabinose are less than 5%. Enzymatic extraction of pectin with the treatment of xylanase to cellulase ratio of 1.5: 1, releases the highest concentration of neutral sugar. High concentrations of neutral sugar have been reported for the enzymatic-ultrasonic extraction of pectin from sisal lesions as well as for the hydrothermal extraction of pectin from sugar beet. High glucose concentrations are mostly related to pectin-bound hydrolyzed cellulose oligomers. As described, cellulose, pectin and hemicellulose are bound together in the cell wall. In addition, previous studies have shown the concentration of glucose or cellobiose in products obtained from the extraction of pectin by acidic method, also the relationship between the concentration of free sugar and the galacturonic column. In general, the optimal purity of the extracted pectin is related to the concentration of galacturonic acid; However, other sugars are also extracted in the process. In fact, proteins ad esterified carboxylic groups in galactron chains have an important

Ratio of xylanase:cellulase enzymes	Sugars content (wt.% based on weight of sample)				Sum of total sugars (wt. %)	Protein content (%)	Ferulic acid content (%)	Degree of esterification (%)	Molecular weight (mol/g)
	GalA	Ara	Xyl + Gal+Rha*	Glc					
1:1	55.73 ± 1.30[a]	0.95 ± 0.031[c]	2.50 ± 0.202[c]	10.12 ± 0.11[b]	69.30	5.6 ± 0.35[a]	0.19 ± 0.013[b]	64.5 ± 5.80[a]	1.47E+05 ± 2.80E+03[a]
1:1.5	58.56 ± 1.23[a]	1.58 ± 0.011[b]	4.08 ± 0.205[b]	16.07 ± 0.84[a]	80.29	5.7 ± 0.14[a]	0.27 ± 0.016[a]	67.7 ± 7.47[a]	1.19E+05 ± 2.83E+03[b]
1:2	50.51 ± 0.76[b]	2.67 ± 0.023[a]	5.59 ± 0.48[a]	11.03 ± 0.44[b]	69.80	6.7 ± 0.26[a]	0.23 ± 0.016[ab]	65.2 ± 8.35[a]	1.21E+05 ± 2.12E+03[b]
Acid-extracted pectin sample**	72.56 ± 0.50	3.01 ± 0.19	4.11 ± 0.20	3.52 ± 0.20	83.20	10.5 ± 0.69	0.49 ± 0.016	60.6 ± 4.33	1.15 E+05 ± 1.97E+03

Calculated as galactose. GalA: galacturonic acid, Ara: arabinose, Gal: galactose, Xyl: xylose, Rha: rhamnose, Glc: glucose.
**Conditions of extraction were: temperature at 85°C, for 2 h, and at pH 2.*
[4], p. 6.

Table 2.
Effect of different doses of cellulase and xylanase enzymes on composition of isolated pectin.

effect on the emulsification properties of pectin. In addition, presence of ferulic acid groups, which are attached to the O-2 position of (1 → 5)-linked arabinose residues in the arabinan side-chains as well as to the O-6 position of galactose residues in (1 → 4)-linked galactans, contributes also to the emulsification efficiency of pectin. **Table 2** shows that enzymatic extraction of pectin from SBP results in pectins with a high degree of esterification (>50%). The different samples of pectin extracted by this method do not show much difference in the ester content. The content of galacturonic acid and ester in this table – continuously – are at a very good level. But the protein content in different samples varied from 5.6% to 6.7%. According to FAO standards, the nitrogen content of pectin should not exceed 2.5%, which refers to 15.6% of protein. The concentration of ferulic acid varies in the range of 0.19% to 0.27% between different samples with a maximum solution of xylanase to cellulase 1: 1.5. Increasing the concentration of cellulase enzyme relative to xylanase from 1: 1 to 1: 1.5 reduces the molecular weight of pectin in the extraction process, while increasing the concentration of cellulase in the extraction solution will not further reduce the weight of pectin molecules. The molecular weight loss of pectin in this case may be due to the presence of pectinase in commercial cellulase enzymes, as these enzymes have the ability to hydrolysis pectin.

Comparison of the chemical composition of pectin obtained from acid extraction in the best conditions (temperature 85°C, 2 h, ph 2) with pectin obtained from enzymatic extraction using a combination treatment with cellulase and xylanase shows a higher concentration of galacturonic acid in the acidic method (72.56%). Also lower glucose content (3.52%), lower ester content (60.6%), lower ferulic acid concentration (0.49%), near arabinose (3.01%), galactose + rhamnose + xylose (4.11%) and protein (5.01%) it shows. The molecular weight of pectin in this case was measured as 1.85E + 05, which shows a higher proportion of pectins from enzymatic extraction in the Abou Elseoud study" [4].

6. What efficacy does the use of different concentrations of enzyme have on the quality of emulsification and the stability of pectin emulsions containing oil in water?

"The pectin that isolated using the different xylanase to cellulase ratios gave close EAI and also stability of emulsion after storage in fridge at 4°C for four weeks. The different emulsions prepared with pectin from different percentages of enzyme concentrations didn't undergo any phase separation. However, after 4 weeks of storage at 4°C, they experienced a decrease in EAI, which was probably the result of the accumulation of emulsion droplets. The yield is almost similar to the emulsification of different samples of enzymatic pectin extracted, probably due to the close protein concentration, and their similar ester content, which plays an essential role in the quality of the emulsion and its stability. In addition, the emulsion extracted from the pectin extracted by the acidic method, EAI showed almost the same as the results obtained by enzymatic extraction. You can compare EAI with other emulsifiers such as soy protein, sodium caseinate and whey protein with EAI of pectin from enzymatic extraction" [4].

7. How does ultrasonic pretreatment affect the performance of pectin from ultrasonic-enzymatic extraction?

"Studies show that the use of ultrasound before treatment with extraction fluid containing enzymes increases the performance of the extracted pectin and also reduces the extraction time. For example, With ultrasonic pretreatment for

15-45 minutes and then treatment with enzymatic extraction fluid for 60 minutes, the yield of pectin shows an increase of about 84-92%. If the enzyme-containing extraction fluid treatment is applied for 120 minutes and the ultrasonic pretreatment is applied for 15-45 minutes, the yield of pectin increases by 67-95%. In addition, if ultrasonic pretreatment is performed for 15-45 minutes and then treated with enzymatic extraction solution for 240 minutes, the pectin yield will not increase significantly (2-16%). The increase in yield of isolated pectin by the ultrasonic treatment is due to the intensification of mass transfer by sonication due to cavitation bubble collapse, which facilitates penetration of the enzymes into the plant tissue because of the increase in porosity and surface area. Ultrasonic applications cause cell swelling and softening of the walls, and by hydrating the pectin in the inner layer, they break down the walls during the process and facilitate the release of pectin" [4].

8. Evaluation of the quality of extracted molecules using ultrasonic-enzymatic technique

"Sugars content was dependent on length of enzymatic treatment time. At the shortest enzymatic time (1 h), increasing ultrasonic treatment from 0 to 45 min resulted in increasing galacturonic acid by about 17%.regarding neutral sugars, the isolated pectin samples after different ultrasonic treatment had close arabinose, rhmanose, galactose, glucose, and xylose contents at the 1-hour enzymatic treatment. At 4-hour enzymatic treatment, ultrasonic treatment time had no effect on galacturonic acid content but arabinose tended to decrease, glucose sugars tended to increase, and no significant effect on rhmanose, galactose, and xxylos was found. There was generally no significant effect for increasing the ultrasonic treatment time on protein content of extracted pectin. Regarding the degree of esterification, pectin extracted using merely enzymatic treatment for 1 h had generally higher degree of esterification than those isolated after 4 h. The effect of ultrasonic treatment time on degree of esterification was depended on enzymatic treatment duration. At 1 h enzymatic treatment, there was no effect for the ultrasonic time on the degree of esterification while in case of 4 h enzymatic treatment the ultrasonic pretreatment resulted in pectin with lower degree of esterification.

Regarding the ferulic acid content, it generally decreased with increasing the ultrasonic pretreatment time in case of the 4-hour enzymatic treatment experiments while no effect was found in case of the 1-Hour enzymatic treatment. This is in accordance with a previous study, which showed that ultrasonic treatment could promote de-esterification of ferulic acid and also methyl and acetyl groups from galacturonic acid units" [4].

9. Evaluation of emulsification quality of pectin extracted by ultrasonic-enzyme method

"Studies show that ultrasonic pretreatment can improve enzyme extraction time. For example, ultrasonic pretreatment for 15-45 minutes and then using enzyme extraction sources for 1 hour, increases the yield of pectin to 92-84%. Also, enzymatic treatment for 2 hours and ultrasonic pretreatment for 15-45 minutes, increases the yield of pectin to 67-95%. However, if the enzyme extraction solution is used for 4 hours and the ultrasonic pretreatment for 15-45 minutes, this number is reduced to 2-16%. Increased yield of pectin extracted by ultrasonic pretreatment

and enzymatic treatment, due to increased mass transfer, occurs during ultrasound through the process of cavity bubble collapse, which itself, due to increased porosity and surface area, will increase the penetration of enzymes into plant tissue. On the other hand, ultrasonic pretreatment, by hydrating the pectin in the inner layer, causes more swelling and softening of the cell wall, and ultimately leads to wall destruction during pretreatment" [4].

10. Therefore...

"Today, we can extract pectin with a very good quality by the extraction solution of cellulase and xylanase enzymes, similar to acid extraction under the best possible conditions. Generally, the benefits of enzymatic extraction far outweigh the benefits of acid extraction. Furthermore, many of the disadvantages of traditional extraction don't exist in green extraction. We must also consider that the enzymatic extraction of pectin in the conditions of using the combined extraction solution of xylanase and cellulase is in its highest quality, which will be much more effective than the method of using single enzymes. In addition, ultrasonic pretreatment in enzymatic extraction method develops and improves pectin processing technology, making extraction time and product quality very desirable. But the use of ultrasonic-enzyme extraction method does not show a significant effect on the emulsification properties of pectins in comparison with enzymatic extraction" [4, 5].

11. Biotechnology ideas for pectin factory solid waste

Since the pectin is extracted from plant products (or pectin-like from algae); Its solid waste can be used to extract or produce valuable materials. One of the ideas for extracting pectin waste is related to substances that can complement your main product. According to studies, if you can extract "cellulose, starch or alginate; You have found a good supplement to increase and improve the effectiveness of your pectin" [11].

Also, if you succeed in commercializing algae to extract pectin-like; You can extract "lipids from its waste to produce biodiesel, carbohydrates to produce bioethanol, protein, or even alginate, and so on" [8, 11–13].

If none of these extractions are cost-effective for your factory, you may welcome the production of fertilizer (algae waste or plant products) and even biochar. If you own a large pectin plant and produce a lot of waste, it may be cost-effective to produce biochar. You can also collect agricultural waste to mix with your waste.

"Biochar, which is produced from biomass pyrolysis, can be used for water's heavy element treatment, such as agricultural fertilizer (with the advantage of reducing the amount of water required), or even as fuel" [6, 14].

12. Biotechnology ideas for pectin factory effluent

Plant effluent can be used to grow algae. "These algae may eventually be used as a source for biofuels, fertilizers, and so on" [8, 12, 13]. "Algae also have the ability to purify water even completely" [15]. "Or you can use these algae to make custom water purification biofilms" [15].

Good luck.

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Chapter 4

Fungal Pectinases in Food Technology

Mohamed Bassim Atta and Fernanda Ruiz-Larrea

Abstract

Pectins contribute to the firmness of plant tissues and confer rigidity to cell walls, protecting the plant from droughts and withering. Fungi have been endowed with an array of extracellular pectolytic enzymes that provide them valuable tools for infecting their host. Pectolytic enzymes are broadly referred to as "pectinases" because they act upon pectin and pectic substances. Pectinases are the most frequently and widely used enzymes in food processing and winemaking. Currently, pectinases are applied in the food industry either to create new products or to improve physicochemical and organoleptic characteristics of conventional products, as well as to increase the yield. This review aims at casting some light on the classification and biochemical aspects of pectinases, especially those of interest in winemaking and food industries. Additionally, it gives a comprehensive summary of current applications of fungal pectinases in the field of food and beverage technology.

Keywords: fungal pectinases, pectic substances, polygalacturonase genes, pectin lyase genes, vegetable food processing, winemaking

1. Introduction

Enzymes are important tools used in the field of food technology, where enzymatic reactions are preferred rather than chemical methods due to the high affinity of enzymes for their substrates, specificity, and that they generate less toxicity. Nowadays, all manufactured food products are prepared using some enzyme at some stage of the production of the food or its ingredients. Different types of enzymes are produced at a commercial scale, which are categorised according to the substrate they act upon. Proteases, lipases, cellulases, and pectinases are the most prevalent of the trade enzymes. Enzymes can also receive different names according to the type of reaction that they catalyse. Thus, lytic enzymes and oxidoreductases are commercialised to be used in the food industry to create new products, or to improve the physicochemical characteristics of conventional products, to enhance their organoleptic properties, and to increase the yield. Among them, pectolytic enzymes, which are broadly referred to as "pectinases", act upon pectin and pectic substances. They are the most frequently and broadly used enzymes in the field of food processing [1] and animal feed. They are also used in paper production and textile industry to degrade the pectic substances that cover

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Company	Web	Brand name	Location
AB Enzymes	http://www.abenzymes.com/	Rohament, Rohapect	Darmstadt, Germany
Advanced Enzyme Technologies Ltd.	http://www.advancedenzymes.com/	SebMash, Seb Enzymes, Seb Enzymes	Chino (CA), USA
AEB (International) Ltd.	http://www.aeb-group.com/	Endozym, Pectizym, Pectocel	Stuttgart, Germany
Apollo Scientific Ltd	https://store.apolloscientific.co.uk/	Macerozyme	Stockport, UK
Biocatalysts Ltd.	http://www.biocatalysts.com/	Pectinase	Cardiff, UK
DSM	http://www.dsm.com/	Rapidase, Crystalzyme, Klerzyme	Delft, The Netherlands
DuPont™ Danisco®	http://www.danisco.com/	Danisco xylanase, Diazyme, Laminex®MaxFlow	Copenhagen, Denmark
Erbslöh Geisenheim AG	http://www.erbsloeh.com/	Fructozym®, Trenolin®	Geisenheim, Germany
Esseco Group	http://www.essecogroup.com/	EnartisZym	San Martino, Italy
Habio	http://en.habio.net/	Food grade xylanases	Sichuan, China
Hubei Hongxin Ruiyu Fine Chemical Co. Ltd.	http://www.hbhxry.com	Pectase	Shanghai, China
Kanto Chemical Co. Inc.	https://www.kanto.co.jp/english/	Polygalacturonase	Chuo-ku, Japan
Laffort	http://www.laffort.com/en	Lafase, Lafazym, Optizym	Bordeaux, France
Lallemand Inc.	http://www.lallemand.com/	Lallzyme	Montreal, Canada
Leveking Enzymes	http://www.levekingenzymes.com/	Xylanase	Shenzhen, China
Maps Enzymes Ltd.	http://www.mapsenzymes.com/	Xylanase, Beta glucanase	Ahmedabad, India
Megazyme International	https://www.megazyme.com/	Pectate lyase, Pectinases	Wicklow, Ireland
Novartis	https://www.novartis.com/	Natuzyme	Basel, Switzerland
Novozymes A/S	http://www.novozymes.com/en/	Novozymes, Pectinex, Ultrazym	Bagsvaerd, Denmark
Shandong Longda Bio-products Co. Ltd.	http://www.longda-enzyme.com/abouten.html	Acid Pectinase, cellulase	Shandong Province, China
Sunson Industry Group Co. Ltd.	http://www.chinaenzymes.com/	Sunson® PEC-pectinase	Yinchuan, China
Tokio Chemical Industry Co. Ltd	https://www.tcichemicals.com/JP/en/	Pectinase from A. niger	Saitama, Japan
Wallerstein Co.	https://www.centerchem.com/products/	Klerzyme	Colorado, USA
Yakult Pharmaceutical Industry Co. Ltd.	http://www.yakult.co.jp/english/	Macerozyme, Pectinase	Tokyo, Japan

Table 1.
Abbreviated list of suppliers of commercial pectinases commonly used in food and beverage industry.

cellulose fibres. Moreover, novel biomedical and drug delivery applications of pectic substances [2] have extended the use of pectinases in the pharmaceutical industry as promising tools to obtain the desired macromolecular product. Commercial enzymes can be obtained from animal tissues, plants, and microorganisms. However, microbial pectolytic enzymes represent about 25% of the total commercial enzymes that are used in food processing [1]. A list of companies that produce commercial pectinases for the food, juice and beverage industries is given in **Table 1**.

There are many attempts to produce pectolytic enzymes on a large scale using different strains of bacteria and fungi. However, *Aspergillus* spp. especially, *Aspergillus niger*, is still the most traditionally used fungus for the production of more than 30 commercial pectolytic enzymes, which are used in the food sector [3] due to its safety status GRAS (Generally Recognized as Safe), according to the USA Food and Drug Administration (FDA) [4].

Although the pectinase market is exposed to fluctuations in the global market the value of industrial pectinase sales in 2019 amounted $30.04 million [5]. The enzyme market is expected to rise further in the near future. Similarly to food enzymes, feed enzymes are gaining increasing importance as they improve the health and performance of livestock. Consequently, development of enzyme production by optimizing fermentation and technique parameters, generation of new strains with a high production via molecular biology and microbial genetics, are current goals of scientists [1].

2. Pectinase substrates

The name "pectinase" indicates that it is an enzyme whose substrate is "pectin." This name is derived from the Greek word "πηκτικός" pēktikós, which means congeal or solidify. Pectin is the descriptive name given to a diverse group of the compounds that are responsible for gel formation. They are normally extracted from fruits and processed to be used, particularly in jams and jellies. Chemically, pectin is an extensive and heterogeneous group of polysaccharides of high molecular weights, whose backbone structure contains galacturonic acid as the main unit. The free carboxylic group of galacturonic acid may be stabilized by divalent ions. Consequently, pectin is generally found in the form of calcium and magnesium pectates. Pectin in native form is present in the primary plant cell wall of dicotyledonous and some monocotyledonous plants [6] as well as in the middle lamella [7]. It may be interlinked with other macromolecules to form insoluble protopectin [8]. The structure and composition of pectic macromolecules depend on the plant source, and some of them may be complex branched heteropolysaccharides containing more than 17 different glycosyl residues, as described in detail in other chapters of this book.

Pectic substances represent about 0.5–4.0% of the weight of fresh plants [9] and maybe expanded to reach 10–30% of the total weight in some plants like turnips, pineapple, tomato pulp, and citrus peels [10]. They contribute to the firmness and structure of plant tissues; they confer rigidity on cell walls [11] and protect the plant from droughts, withering, hazardous microorganisms, and an array of other plant pathogens.

Pectic substances could be taken as indicators of maturity and ripening of vegetable and fruits during growing as well as evolution of texture through storage. Nutritionally, insoluble pectic substances of fruits and vegetables are a significant part of the dietary fibre, which provides the beneficial effect of protecting consumers from chronic diseases, especially diabetes and colorectal cancer [12, 13].

As for the soluble pectic substances, which are normally extracted from agro-industrial by-products, such as citrus and apple peels are traditionally used as gelling and/or thickening substances in the field of food processing and of other industries.

The main unit in the chemical structure of the pectin and pectic substances is α-D-galacturonic acid which is linked by (1 → 4) bonds. The side chains of the pectic macromolecule may include even 17 different types of monosaccharides, of which α-L-arabinofuranose, α-D-galactopyranose, α-L-rhamnopyranose, and β-L-xylofuranose are the most abundant units. As a matter of fact, pectic substances include two different high molecular weight fractions of polysaccharides: homo- and hetero-polysaccharides, and thus, according to their monosaccharide composition and structure, pectic substances can be classified following a simplified system into the following main groups: homogalacturonans, rhamnogalacturonans type I, rhamnogalacturonans type II, xylogalacturonans, and other heterogalacturonans.

Homogalacturonan. The homopolysaccharide homogalacturonan (**HG**) is a linear chain of (1 → 4) linked α-D-galactopyranosyluronic acid (*Galp*A) residues in which some of the carboxyl groups are either methyl esterified or O-acetylated at C3 or C2 depending on the plant of pectin source. As homogalacturonans are linear polysaccharide chains, they could be described as "smooth" regions of pectic macromolecules.

Rhamnogalacturonan-I. The heteropolysaccharide group named rhamnogalacturonan-I **(RG-I)** includes pectic substances with a backbone structure consisting of the repeating disaccharide [(1 → 4)-α-D-*Galp*A-(1 → 2)-α-L- *Rhap*-(1→)] where α-L-*Rhap* stands for α-L-rhamnopyranose. The backbone of *Galp*A residues may be O-acetylated on C-2 and/or C-3 [14]. There is no conclusive chemical evidence that the *Galp*A residues are methyl esterified, however, an enriched RG-I-like wall fraction from flax has been reported to contain methyl esters [15].

Rhamnogalacturonan-II. Rhamnogalacturonan-II (**RG-II**) is a group of non-soluble pectic polysaccharides found normally in plant cell walls that are solubilised by treating the cell wall with endopolygalacturonases. They have molecular weights ranged between 5 and 10 kDa. The backbone of RG-II contains at least eight repeating units of 1 → 4-linked α-D-*Galp*A residues and it is substituted with highly complex side chains that contain at least 12 different glycosyl residues [16]. Two structurally distinct disaccharides (chains C and D) are attached to C-3 of the backbone and two structurally distinct oligosaccharides (chains A and B) are attached to C-2 of the backbone (**Figure 1**).

Depending on the plant source and the method of isolation, between 20 and 80% of the *Rhap* residues are substituted at C-4 with neutral and acidic oligosaccharides. The oligosaccharides contain linear and branched α-L-arabinofuranose and β-D-galactopyranose residues. Some of these side chains may be terminated with α-L-fructopyranose, β-D-glucuronic acid, and 4-O-methyl β-D-glucuronic acid residues [18, 19].

Other heterogalacturonans. Among heterogalacturonans xylogalacturonans (**XG**) have been gaining more attention and found mainly in fruit pectins [20]. They have a homogalacturonan backbone with frequent single xylose residues linked β-(1 → 3) to about half of the galacturonic acid residues.

Rhamnogalacturonans I and II and xylogalacturonans are branched-chain polysaccharides, and some authors describe them as "hairy" regions of pectic macromolecules [2].

As mentioned above, this classification is made according to the monosaccharide composition and structure of the pectic substances. Nevertheless, pectic substances can be also grouped into four categories according to their molecular weight, water-solubility, and degree of methoxylation of their carboxylic acid groups, namely:

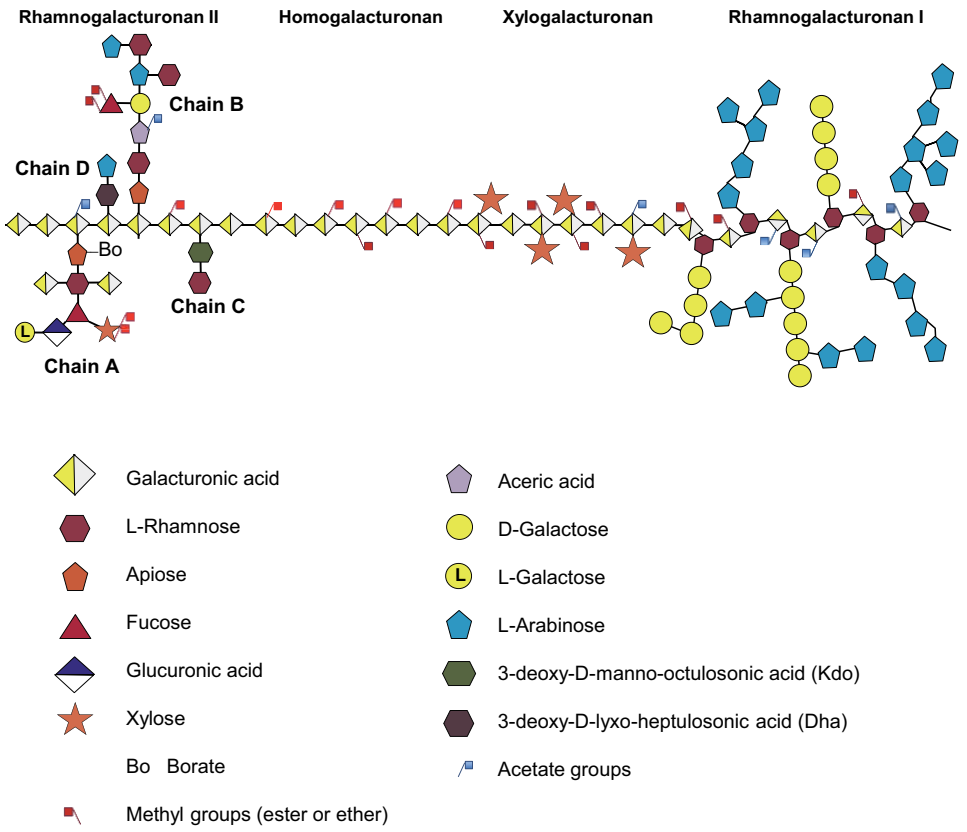

Figure 1.
Model structure of pectin as methyl ester polygalacturonic acid branched at RGI, RG-II, and XG (modified from [17]).

pectic acid, pectinic acid, pectin, and protopectin [8]. Whereas protopectin is water-insoluble, the other three are either totally or partially soluble in water.

Protopectin is the parent form of pectic substances and upon restricted hydrolysis yields pectin, pectinic, and pectic acids, as well as other derivatives. It is the term used to describe the native water-insoluble pectic substances found in the plant cell wall and middle lamella from which soluble pectic substances are produced.

Pectic acid is a product of the hydrolysis of pectin it is mainly polygalacturonic acid in the form of a linear polymer of the repeating unit of galacturonic acid, which contains negligible amounts of methoxyl groups. Free carboxylic groups could be partially or completely neutralised by sodium, potassium, or ammonium ions. Salts of pectic acid are called pectates.

Pectin represents the main carbohydrate component of the primary cell wall and middle lamella which accounts for about one-third of the total cell wall material [21]. As mentioned above, the main unit of pectin is galacturonic acid esterified at its carboxylic group with methanol, and the methoxylation ratio of galacturonic acid residues fluctuates between 60 and 90%.

Pectinic acid is the intermediate in methyl ester content between pectic acid and pectin ($0 \leq$ methoxylation ratio $< 60\%$). Pectinates is a common name of pectinic acid salts.

The molecular weights of pectic substances range from 25 to 360 kDa and their degree of methoxylation fluctuates depending on the plant source and method of extraction. Free hydroxyl groups of galacturonic acid are partly or completely

neutralised by sodium, potassium, or ammonium ions, and some of the hydroxyl groups on C2 and C3 may be acetylated [22].

3. Pectolytic enzymes and their classification

Pectolytic enzymes constitute a wide group of enzyme activities that are responsible for the degradation of the pectic substances summarised in the previous section. During fruit ripening, endogenous pectolytic enzymes act upon insoluble pectic substances and turn them into soluble pectic substances. As a result, the surrounding cell wall loses its grip and firmness, and consequently the plant tissue softens. Analogously, plant pathogens attack their host by secreting many different pectolytic enzymes in addition to cellulases and proteases [23]. This means that pectolytic enzymes exist in different forms depending on the source, the substrates they act on, and the products to be split from the substrate. Generally, pectolytic enzymes are divided into two groups: depolymerizing enzymes and esterases (also named saponifying enzymes) [24]. Depolymerizing enzymes can cleave the α-(1 → 4) glycosidic bonds in the backbone of the pectin chain, and in this group polygalacturonase (**PG**), pectin lyase (**PNL**), and pectate lyase (**PL**) are included. While pectin methyl esterases (**PE**) (commonly named "pectin esterases") and pectin acetyl esterase (**PAE**) break down ester linkages splitting methoxyl or acetyl groups, liberating the carboxylic groups of pectin polygalacturonic acid residues.

Classification of the plethora of pectolytic activities and their correct naming can be achieved following the consensus recommendations of the International Union of Biochemistry and Molecular Biology [25] and thus, pectolytic enzymes can be grouped as follows:

I. **Glycosidases** (*EC 3.2.1*) that hydrolyse O-glyosidic bonds, among which **polygalacturonases** are the main enzymes acting on pectic substances (**Figure 2**). These enzymes catalyse the hydrolysis of α-(1 → 4) glycosidic linkages in galacturonans. There are three types of these enzymes:

   a. *Endo*-polygalacturonase (***endo*-PG** *EC 3.2.1.15*) catalyses random hydrolysis of α-(1 → 4) glycosidic linkages in pectates and other galacturonans. Other names of this enzyme: polygalacturonase, *endo*-galacturonase, pectin-depolymerase, pectinase; pectolase, pectin-hydrolase, and *endo*-polymethylgalacturonase (*endo*-PMG).

   b. *Exo*-polygalacturonase (***exo*-PG** *EC 3.2.1.67*) catalyses the hydrolysis in a sequential cleavage of the α-(1 → 4) glycosidic linkage of the

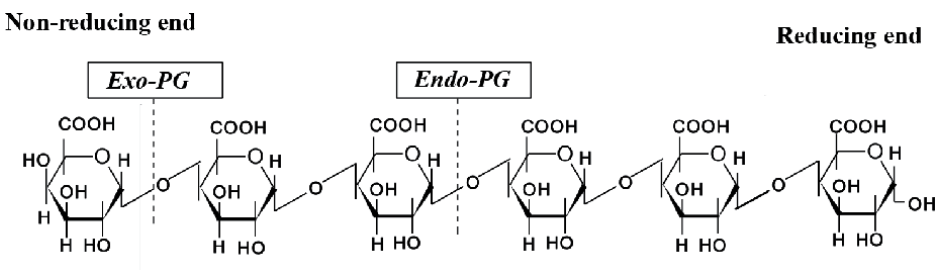

Figure 2.
Sites of hydrolysis of α-(1 → 4) glycosidic linkages catalysed by polygalacturonase enzymes.

non-reducing end of pectates and other galacturonan chains. Other names: galacturan 1,4-α-galacturonidase, poly [(1 → 4)-α-D-galacturonide] galacturonohydrolase, poly(galacturonate) hydrolase, *exo*-D-galacturonase, poly (1,4-α-D-galacturonide) galacturonohydrolase, and *exo*-polymethylgalacturonase (*exo*-PMG).

c. *Exo*-polygalacturonan-digalacturono hydrolase (*EC 3.2.1.82*) is a very specific activity that catalyses the hydrolysis of pectic acid from the non-reducing end, releasing digalacturonate. Other names of this enzyme: poly [(1 → 4)-α-D-galactosiduronate] digalacturonohydrolase, exopolygalacturonosidase, and others.

d. There are some other glycosidases in the *EC 3.2.1* group that can hydrolyse O- glycosidic bonds in rhamnogalacturonans and other heteropolysaccharides, such as β-galactosidase (*EC 3.2.1.23*), non-reducing end α-L-arabinofuranosidase (*EC 3.2.1.55*), non-reducing end β-L-arabinopyranosidase (*EC 3.2.1.88*), arabinogalactan *endo*-β-1,4-galactanase (*EC 3.2.1.89*), arabinan *endo*-1,5-α-L-arabinanase (*EC 3.2.1.99*), rhamnogalacturonan hydrolase (*EC 3.2.1.171*), xylan 1,3-β-xylosidase (*EC 3.2.1.72*), rhamnogalacturonan galacturonohydrolase (*EC 3.2.1.173*), and rhamnogalacturonan rhamnohydrolase (*EC 3.2.1.174)*. These glycosidases cooperate in degrading side chains, but their role is not as relevant as that of polygalacturonases for the degradation of pectic molecules.

II. **Polygalacturonan lyases** are a group of enzymes that cleave α-(1 → 4) glycosidic linkages by trans-elimination or ß-elimination (*EC 4.2.2*) to give oligosaccharides with an unsaturated bond between C4 and C5 at their non-reducing ends (**Figure 3**). There are several subclasses for these enzymes:

a. Pectin lyases **(PNL** *EC 4.2.2.10***)** catalyse the eliminative cleavage of α-(1 → 4) glycosidic linkages in pectins. Other names: pectin trans-eliminase, *endo*-pectin lyase, polymethylgalacturonic transeliminase, pectin methyltranseliminase, pectolyase, polymethoxygalacturonide lyase, and polymethylgalacturonate lyases (PMGL). They prefer to act upon highly esterified pectins without the prior action of other enzymes [26] and demethylation of pectins progressively slows their activity. Two activities can be included under this denomination: *endo*-pectin lyase, which comprises most of the studied pectin lyases (*endo*-PNL = *endo*-PMGL) and *exo*-pectin lyase (*exo*-PNL = *exo*-PMGL), which includes scarcely reported enzymes [27].

b. Pectate *endo*-lyases (***endo*-PL** *EC 4.2.2.2*) catalyse the cleavage of α -(1 → 4) glycosidic linkages in pectic acid and pectates. They show specificity for pectates in their anion form over methyl esterified pectins. Other names: *endo*-pectate lyase; polygalacturonic transeliminase; pectic acid transeliminase; polygalacturonate lyase; pectic lyase; α-1,4-D-*endo* polygalacturonic acid lyase, and others. This enzyme type is usually employed for de-gumming natural fibres in the paper and textile industries [28]. It is also used for the preparation of fruit and vegetable maceration products and agriculture wastewater treatment [9].

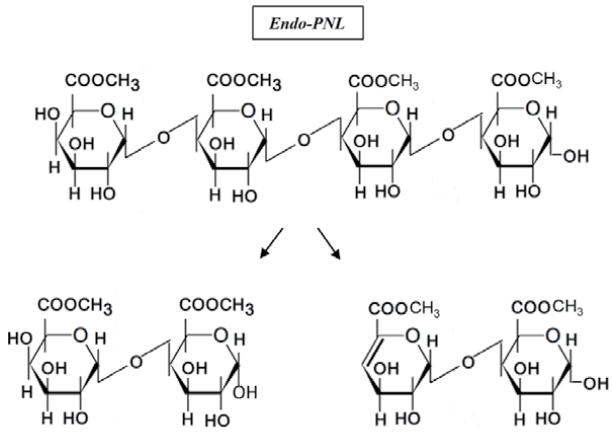

Figure 3.
Endo-*pectin lyase catalyzed reaction*

c. Pectate *exo*-lyases (disaccharide-lyase *EC 4.2.2.9*; trisaccharide-lyase *EC 4.2.2.22*) that release di- and tri-saccharides upon cleavage of pectic acid and pectates.

d. Oligogalacturonide lyase (*EC 4.2.2.6*) cleaves a digalacturonate derivate to render two oxidised monosaccharides.

e. Other polysaccharide lyases that cleave rhamnogalacturonans (rhamnogalacturonan-*endo* lyase *EC 4.2.2.23* and rhamnogalacturonan-exo lyase *EC 4.2.2.24*) (IUBMB, 2021).

III. **Esterases** that hydrolyse carboxylic ester linkages (*EC 3.1.1*) in galacturonans include some subclasses:

a. Pectin esterase **(PE** or PME *EC 3.1.1.11***)** catalyses the hydrolysis of the ester linkage between the methoxyl group and the carboxylic group of galacturonic acid residues in the pectin or pectinic acid backbone, releasing methanol (**Figure 4**). Other names: pectin methylesterase (PME); pectin demethoxylase; pectin methoxylase; pectase and pectinesterase.

The presence of calcium ions maintains fruit firmness by binding to free negatively charged carboxylic acid groups of the pectin molecules that are not methoxylated, forming what is defined as the "egg-box" model with a structure of calcium ion cross-bridges between pectin chains [29]. In this regard, the activity of the pectin esterase can play a role in fruit texture [30].

b. Pectin acetyl esterase (**PAE** *EC 3.1.1.6*) splits the acetyl group from pectin. The enzyme acts preferentially on the ester linkage of a galacturonate unit next to a non-esterified galacturonate unit [8].

c. Other carboxyl-esterases, like rhamnogalacturonan acetylesterase (*EC 3.1.1.86*) that hydrolyses acetyl groups in type I rhamnogalacturonans.

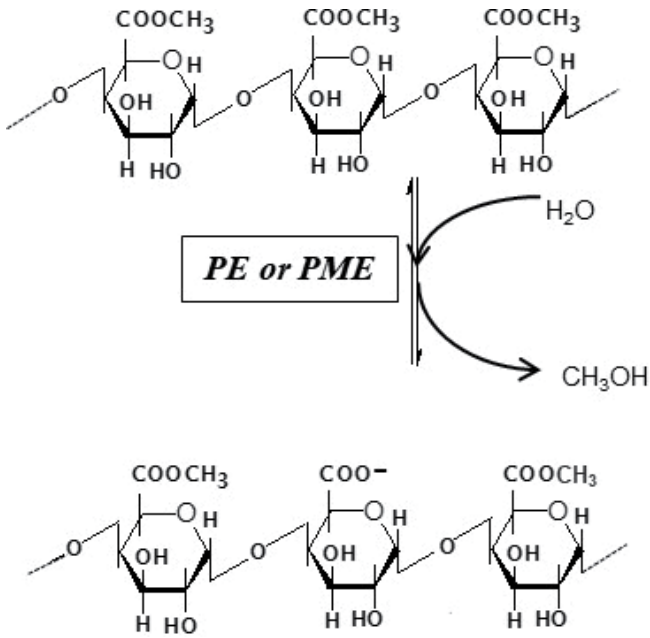

Figure 4.
Pectin esterase (EC 3.1.1.11) catalysed hydrolysis of the methoxy group of pectin to yield methanol.

IV. **Protopectinases.** A mixture of some of the previous enzymes besides other polysaccharidases, such as cellulase or hemicellulase, and protease, can act on the water-insoluble protopectin aggregates, turning them into highly water-soluble pectic substances [9]. This heterogeneous group of enzymes that act on protopectin aggregates is commonly known as protopectinases.

4. Fungal pectolytic enzymes

Fungi secrete pectolytic enzymes into their growth medium in combination with some other polysaccharide-degrading enzymes, like cellulase, hemicellulase, amylase, and other extracellular secreted enzymes, such as proteases. All these enzymes play an important role in infecting host cells by filamentous fungi. From the biotechnological point of view, extracellular enzymes are easier to obtain than intracellular enzymes, as secreted enzymes are recovered in the culture broth supernatant and simultaneously separated from the remaining cellular biomass of the producer organism. These reasons make filamentous fungi excellent candidates for enzyme production. Nevertheless, in order to use fungal enzymes in the industrial sector, stability and biochemical characteristics of such enzymes produced under various growing conditions must be studied. Purification of an enzyme is needed to estimate its biochemical properties and specificity. On the other hand, the numerous steps that are usually required for the complete purification of an enzyme, consume a long time, largely increase the economic cost and resources. In addition, the purification process could have negative effects on the enzyme activity. Consequently, the balance between technical and economic requirements is mandatory for the industrial production of enzymes.

There are different methods and techniques to separate and isolate pectolytic enzymes from crude extracts. These methods are diverse in their efficiency

and resolution. Precipitation of the enzyme from crude extracts using natural salts (e.g., ammonium sulphate) or organic solvent (e.g., ethanol) followed by column chromatography is a satisfactory procedure to get commercial purified enzymes [10].

One of the early attempts to purify pectolytic enzymes was that of two *exo*-PG isolated from crude extracts of *A. niger* after DEAE-cellulose chromatography, using 0.2 M sodium acetate buffer at pH 4.6 as the eluting solvent. The specific activity of both enzymes was increased 209- and 205-fold with 8.6–1% recovery, respectively [31]. PG from *Rhizopus stolonifer* was also separated by ethanol precipitation followed by CM-Sepharose 6B ion-exchange chromatography and the eluate was further purified to reach 10-fold by gel filtration onto Sephadex G-100 [32]. PG and PNL from *Aureobasidium pullulans* LV10 were separated by CM-Sepharose 6B followed by DEAE-cellulose chromatography and gel filtration on Sephadex G-100 [33]. *Endo*-PG from Rohament P, a commercial pectolytic enzyme from *A. niger*, was isolated and separated into three isoenzymes by preparative isoelectric focusing onto Bio-gel P-60 [34]. Here it should be noted that multiple purification steps will elevate the enzyme price, thereby, researchers in the field of food processing should pay great attention to make the purification method easy, fast, and inexpensive while increasing as much as possible the enzyme activity.

Immobilised metal ion affinity polysulphone hollow-fibre membranes with a high capacity for protein adsorption were successfully used to separate PNL and PE from a commercial pectolytic preparation [35]. A rapid and simple method to separate pectinases, including PE and PG, from potato enzyme preparations using perfusion chromatography (Poros HS), was introduced by Savary [36]. This method was an economical purification strategy for PE and PG enzymes from crude extract at a commercial scale. Regarding cold-active pectinases isolated from psychrotolerant yeasts, few activities have been characterized that showed pectolytic activity at low temperatures, reaching down to 5°C [37].

The term "pectinase" is widely preferred in the industrial context, and a wide range of pectinase-producing fungi and procedures for recovering and purifying the enzyme can be found in literature and have been successfully applied for industrial purposes [1, 3, 38]. In addition, the production of recombinant pectinases by genetically manipulated fungi has gained the attraction of researchers and biotechnologists [3, 39]. Nevertheless, as mentioned above, the GRAS status awarded by the FDA to the enzyme producer organism is a relevant characteristic when enzyme production is intended for the food industry. **Table 2** shows fungal producers of pectinolytic enzymes that are currently included in the inventory of GRAS notices, being *Aspergillus* the most repeated genus in the GRAS inventory, and *A. niger* the species with the highest number of notices, as mentioned above. With regard to the use of enzymes in foods in the European Union, it is subject to the legislation of its member states, and currently, the European Food Safety Authority (EFSA) is in the process of evaluating the safety of more than 300 food enzymes, whose applications were submitted for approval to be included in a future EU list of authorised food enzymes [40].

5. Pectolytic enzymes from fungi and yeast with GRAS status

Tables 3 and **4** show polygalacturonases (*EC* 3.2.1.15) and pectin lyases (*EC* 4.2.2.10) from fungal and yeast species that possess the GRAS status and that can be currently found at the NCBI Protein Database. **Table 3** shows the

	Species
Filamentous fungi	
	Aspergillus niger
	Aspergillus oryzae
	Aspergillus tubingensis
	Candida rugosa
	Candida cylindracea
	Disporotrichum dimorphosporum
	Humicola insolens
	Leptographium procerum
	Penicillium camemberti
	Penicillium chrysogenum
	Penicillium funiculosum
	Rhizopus oryzae
	Trichoderma harzianum
	Trichoderma reesei
Yeast	
	Kluyveromyces lactis
	Kluyveromyces marxianus
	Saccharomyces cerevisiae

Table 2.
Filamentous fungi and yeast species producers of pectinolytic enzymes that are included in the GRAS inventory of the FDA.

exo-polygalacturonase encoding genes found in fungi: pgxA, pgxB, and pgxC, and those that encode *endo*-polygalacturonases, which are quite numerous: pgaA, pgaB, pgaC, pgaI, and pgaII; whereas only the pgU1 gene was found in GRAS yeast strains. All the polygalacturonases encoded by these genes belong to the glycosyl hydrolases family 28.

The genes that encode pectin lyases found in GRAS fungi are quite numerous as well and pectin lyases from *Aspergillus* strains have been characterised according to their substrate degradation profile [41]. **Table 4** shows the pectin lyase encoding genes that have been sequenced from GRAS fungi: pel1, pel2, pelA, pelB, pelC, pelD, pelE, and pelF. All the encoded proteins belong to the polysaccharide lyase family 6. It is worth mentioning that no pectin lyase has been described in GRAS yeast.

The protein structure of the *endo*-polygalacturonase II of *A. niger* was determined by crystallographic techniques [42] and its sequence is 60% identical to the *endo*-polygalacturonase I. The 1.70 Å resolution crystal structure of *endo*-polygalacturonase I is shown in **Figure 5**. It is folded into a right-handed parallel beta helical structure comprising 10 complete turns. This structure includes a narrow substrate-binding cleft, in which the Arg96 residue, previously shown to be critical for the enzyme activity, was shown to interact with the polygalacturonic acid units of the backbone of its substrate [43].

The protein structure of the pectin lyase A and pectin lyase B of *A. niger* were as well resolved in the 90s [44, 45]. *A. niger* pectin lyases shown in **Table 4** share

Species	Strain	Protein ID	Protein length (aas)	Protein name (gene name)
Aspergillus niger	ZJ5A	AQT01640.1	362	endo-polygalacturonase
Aspergillus niger	CBS 101883	XP_025459364.1	370	endo-polygalacturonase A (pgaA)
Aspergillus niger	ATCC 9029	sp|Q9P4W3.1	362	endo-polygalacturonase B (pgaB)
Aspergillus niger	CBS 101883	XP_025460991.1	362	endo-polygalacturonase B (pgaB)
Aspergillus niger	CBS 513.88	XP_001399628.1	362	endo-polygalacturonase C
Aspergillus niger	CBS 513.88	XP_001390812.1	384	endo-polygalacturonase C
Aspergillus niger	CBS 101883	XP_025455246.1	378	endo-polygalacturonase C
Aspergillus niger	CBS 101883	XP_025455528.1	368	endo-polygalacturonase I (pga1)
Aspergillus niger	N400	CAA41693.1	368	endo-polygalacturonase I (pga1)
Aspergillus niger	RH 5344	P0CU55.1	362	endo-polygalacturonase II (pgaII)
Aspergillus niger	An15c0180	CAK42510.1	362	endo-polygalacturonase II (pgaII)
Aspergillus niger	CBS 513.88	CAK42510.1	362	endo-polygalacturonase II (pgaII)
Aspergillus niger	CBS 101883	XP_025453406.1	362	endo-polygalacturonase II (pgaII)
Aspergillus niger	CBS 101883	PYH57102.1	434	exo-polygalacturonase A (pgxA)
Aspergillus niger	CBS 513.88	ABD61563.1	434	exo-polygalacturonase A (pgxA)
Aspergillus niger	CBS 513.88	ABD61564.1	438	exo-polygalacturonase B (pgxB)
Aspergillus niger	CBS 513.88	ABD61565.1	440	exo-polygalacturonase C (pgxC)
Aspergillus niger	CBS 513.88	ABD61562.1	435	exo-polygalacturonase X
Aspergillus oryzae	PO	AHA43015.1	367	endo-polygalacturonase
Aspergillus oryzae	3042	EIT75145.1	367	endo-polygalacturonase C
Aspergillus oryzae	100-8	KDE81298.1	367	endo-polygalacturonase C
Aspergillus oryzae	KBN616	BAA03244.2	363	polygalacturonase
Aspergillus tubingensis	NW756	CAA41695.1	362	endo-polygalacturonase II (pgaII)
Kluyveromyces marxianus	DMKU3-1042	XP_022674029.1	394	endo-polygalacturonase (pgu1)
Penicillium chrysogenum	P2niaD18	KZN90643.1	369	polygalacturonase
Rhizopus oryzae	NRRL 29086	ACA48699.1	383	polygalacturonase
Rhizopus oryzae	YM9901"	BAD67423.1	383	polygalacturonase
Saccharomyces cerevisiae	S288C	NP_012687.3	361	endo-polygalacturonase (pgu1)
Saccharomyces cerevisiae	YJM693	AJR55394.1	361	endo-polygalacturonase (pgu1)
Saccharomyces cerevisiae	YJM1208	AJR66759.1	376	endo-polygalacturonase (pgu1)
Trichoderma harzianum	T6776	KKO99256.1	457	endo-polygalacturonase
Trichoderma reesei	QM6a	XP_006966948.1	401	polygalacturonase
Trichoderma reesei	QM6a	XP_006969524.1	401	polygalacturonase

Table 3.
Polygalacturonases (EC 3.2.1.15) included in the NCBI Protein Database that were obtained from fungal and yeast GRAS species.

Species	Strain	Gene name	Protein ID	Protein length (aas)	Protein name
Aspergillus niger	FDAARGOS_311	pel1	TPR10550.1	215	pectin lyase 1
Aspergillus niger	SC323	pelA	AKA88528.1	379	pectin lyase A
Aspergillus niger	CBS 513.88	pelA	CAK48529.1	379	pectin lyase A
Aspergillus niger	N400	pelA	CAA43130.1	379	pectin lyase A
Aspergillus niger	EIM-6	pelA	AFJ80127.1	370	pectin lyase A
Aspergillus niger	EIM-7	pelA	AFJ80126.1	371	pectin lyase A
Aspergillus niger	ZJ5	pelA	ALB05716.1	379	pectin lyase A
Aspergillus niger	CBS 513.89	pelB	CAK37997.1	379	pectin lyase B
Aspergillus niger	CBS 101883	pelB	PYH55243.1	379	pectin lyase B
Aspergillus niger	ATCC13496	pelB	RDH19663.1	379	pectin lyase B
Aspergillus niger	CBS 101883	pelB	XP_025453298.1	379	pectin lyase B
Aspergillus niger	N400	pelB	CAA46521.1	378	pectin lyase B
Aspergillus niger	N400	pelC	AAW03313.1	378	pectin lyase C
Aspergillus niger	ZJ5	pelC	AIX03726.1	475	pectin lyase C
Aspergillus niger	CBS 513.88	pelD	XP_001402523.3	373	Pectin lyase D
Aspergillus niger	CBS 513.88	pelD	CAK47350.1	373	pectin lyase D
Aspergillus niger	N756	pelD	AAA32701.1	373	pectin lyase D
Aspergillus niger	MTCC:404	pelD	AIE38009.1	373	pectin lyase D
Aspergillus niger	CBS 120.49	pelE	ACE00421.1	370	pectin lyase E
Aspergillus niger	An76	pelE	GAQ35327.1	382	pectin lyase E
Aspergillus niger	An76	pelF	GAQ35503.1	379	pectin lyase F
Aspergillus niger	An76	pelF	GAQ40247.1	475	pectin lyase F
Aspergillus niger	CBS 513.89	pelF	XP_001389926.1	379	pectin lyase F
Aspergillus niger	CBS 120.49	rglA	CAD36194.1	499	rhamnogalacturonan lyase A
Aspergillus oryzae	KBN616	pel1	BAB82467.1	381	pectin lyase 1
Aspergillus oryzae	KBN616	pel 2	BAB82468.1	375	pectin lyase 2
Aspergillus oryzae	RIB40	pel2	Q2TXS4.1	375	pectin lyase 2
Aspergillus oryzae	100–8	pelF	KDE86143.1	381	pectin lyase F
Aspergillus tubingensis	WU-2223 L	pelB	XP_035354767.1	379	pectin lyase B
Aspergillus tubingensis	WU-2223 L	pelD	XP_035361751.1 GFN20947.1	373	pectin lyase D
Humicola insolens	Y1		QEI10431.1	248	pectate lyase
Penicillium camemberti	FM013		CRL23631.1	241	pectate lyase

Table 4.
Pectin lyases (EC 4.4.4.10) included in the NCBI protein database that were obtained from fungal and yeast GRAS species.

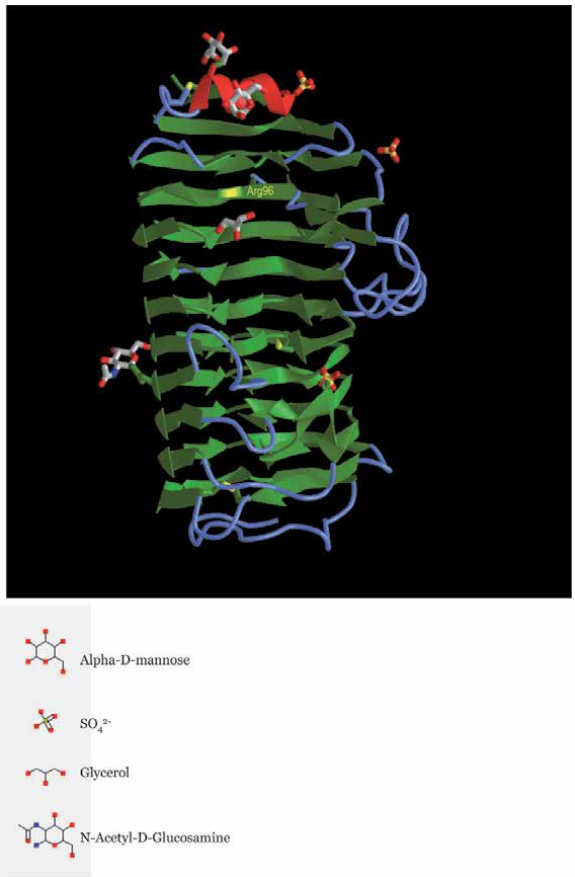

Figure 5.
PDB Image of Endo-*polygalacturonase I from A. niger at 1.7 Å resolution [43]. Available from: https://www.ncbi.nlm.nih.gov/Structure/pdb/1NHC. Code of colours: Beta strands in green, loops in blue, alpha hélix in red.*

46–65% amino acid sequence identity. The 1.70 Å resolution crystal structure of pectin lyase B is shown in **Figure 6** [45] and it shows a parallel beta helical structure, where residues Asp154, Arg176, and Arg236 were expected to play a role in the catalysis [44]. In contrast to the previously shown structure of *endo*-polygalacturonase, the pectin lyase structure shows a number of loops of various sizes and conformations that protrude from the central helix, which bind oligosaccharides and probably confer function to the enzyme [45].

6. Applications of acidic pectinases in food technology

Pectolytic enzymes can be grouped into two categories according to their optimum pH values. The first one includes acidic pectinases that have optimum activity at pH 3.0–5.5 and 30–50°C. This group of enzymes is generally secreted by fungi, especially *Aspergillus sp*., and rarely secreted by bacteria. The second group is alkaline pectinases, which are typically bacterial pectinases and rarely secreted by fungi. The optimum pH values for this group of enzymes fluctuate from 8.0 to 10.5, while optimum temperatures are ranged from 45 to 75°C [46].

In the field of food technology, acidic pectinases are routinely used to increase the proportion of juice extraction by mechanical pressing or crushing [9, 47, 48], to increase filtration efficiency and clarification of fruit juice. These are mandatory operations for fruits and vegetables rich in pectic substances such as citrus and

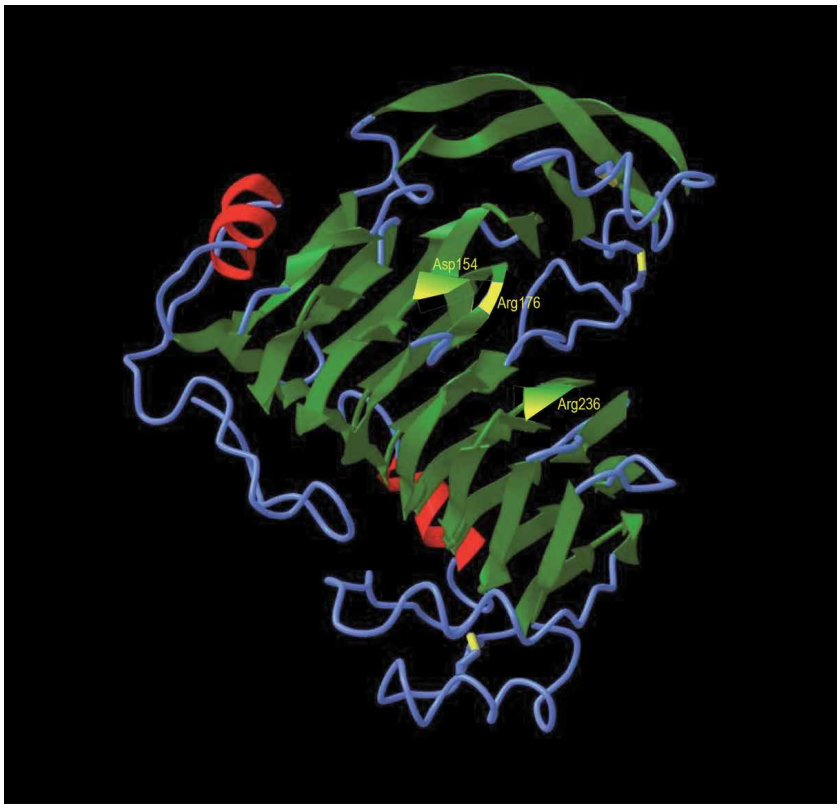

Figure 6.
PDB Image of Pectin Lyase B from A. niger at 1.7 Å resolution [45] Available from: https://www.ncbi.nlm.nih.gov/Structure/pdb/1QCX. Code of colours: Beta strands in green, loops in blue, alpha hélix in red.

tomato, as well as for liquefaction. Specialised pectolytic enzymes that act only upon the middle lamella (macerating enzymes) can be used to prepare products focused on preserving the integrity and shape of the plant cells. These enzymes keep the pulp juice with the taste and flavour of the original fruit. Pectinases can also be used in protoplast fusion technology [49]. Therefore, a wide variety of objectives that require the addition of pectolytic enzymes have been reported according to the purpose of the technological process.

7. Extraction of vegetable and fruit juice

In the course of juice preparation from fresh fruits with mechanical crushing or pressing, a soluble proportion of pectic substances (water-soluble pectin) is released in the liquid phase leading to an increase of the juice viscosity. However, insoluble pectic substances remain bound to hemicellulose and cellulose fibrils by means of side chains [50]. Water is retained with pulp particles leading to hinder the flow of cell sap, which remains bound to the pulp in the form of a jellified mass. Consequently, the juice yield is low. Also, raw press juice contains insoluble pectin particles (cloudy particles) that carry surface negative charges, which can coat positively charged surface proteins forming particles that give unpleasant mouth-feeling and off-tastes for many consumers. A mixture of pectolytic enzymes with cellulase could be useful to degrade both pectin and cellulose of cell walls and middle lamella by breaking down the pectin chain and other attached polysaccharides to their mono- and oligo-monomers. As a result, a crystal-clear juice with a

good appearance, low viscosity, high stability, appropriate mouth-feeling, and taste characteristics will be obtained, in addition to the benefit of an increase in the yield. Pectinase treatments are usually performed to prepare juice from fruits containing a high percentage of pectic substances, such as apple, pear, berries, citrus, and banana [51]. A mixture of pectinases can also decrease the filtration time up to 50% [52]. However, in some cases, it is necessary to use a mixture of pectinases, cellulases, arabinases, and xylanases to increase the fruit pressing efficiency for juice extraction [53]. In this respect, Josh *et al.* [54] found that a partially purified pectinase, produced by *A. niger* in solid-state fermentation of apple pomace, could increase juice extraction from 52 to 78% in plum, 38 to 63% in peach, 60 to 72% in pear, and 50 to 80% in apricot. The advantages of pectinase addition are: increase of colour, titrable acidity, total sugars in the extracted juices, decrease of pH, Brix/acid ratio, and relative viscosity. Biochemical properties of some microbial pectinases, including those produced by bacteria, can be found in a former review [39].

Mixtures of pectinases, cellulases, and hemicellulases can be used to assist in the extraction of edible oils, thus to increase the extraction efficiency of vegetable oils. The mixture of enzymes that hydrolyze complex polysaccharides of the cell walls of oilseeds and oily fruits, liquefies the structure of cell walls, leading to the release of the sap of cells including oil and fat-soluble active molecules (e.g., α- and β-carotenes, sterols, and vitamin D) that prevent oxidation of oils.

8. Liquefaction and stabilisation of juice

From the chemical point of view, liquefaction is the process of converting a substance from its solid or gas phase into a liquid phase. As for food technology, liquefaction means the process of turning insoluble macroparticles of tissues into smaller and soluble particles via degrading enzymes [55]. Vacuum infusion of pectinases [56] has a commercial application to soften the peel of citrus fruits.

Nectars are cloudy fruit juices mixed with syrup and citric acid to produce a ready-to-drink beverage. A serious defect of nectars is the precipitation of the cloudy particles in the bottom of the container, forming a gel and leaving a clear supernatant layer, which consumers decline. The addition of *exo*-pectinases could

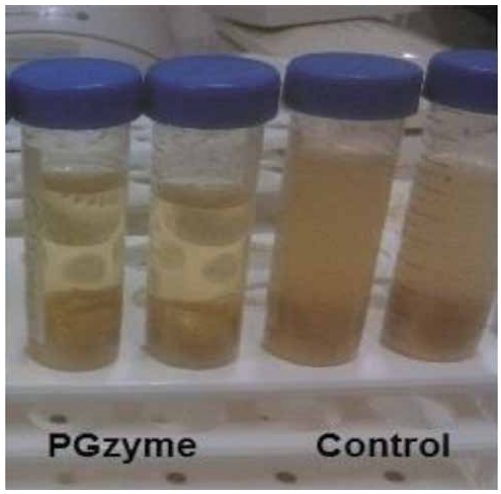

Figure 7.
Effect on banana juice of the pectinase extract PGzyme produced by Aspergillus sojae (ATCC 20235) on Banana juice as clarification and stabilization enzyme [57].

improve the stability of cloudy particles and render stable homogeneous nectar. Commercial enzymes that have high PG and PNL activities combined with cellulases and hemicellulases are used to decrease the viscosity and to keep cloud stability. In this respect, a crude extract that contained a mixture of pectinases (exo-PG, endo-PG and PNL) produced by *Aspergillus sojae* ATCC 20235, which was named PGzyme, was successfully employed to clarify a cloudy banana juice. As a result, the cloudy juice was converted into a crystal stable translucent drink (**Figure 7**) [57].

9. Food texture

Maceration is a process by which an organised tissue is transformed into a suspension of intact cells, resulting in pulpy products (soft texture), which are used as a base material for pulpy juices nectar, baby foods, and ingredients for dairy products, such as puddings and yogurts [39]. Enzymatic degradation of pectin after a mild mechanical treatment often improves the properties of the final product. An appropriate treatment with pectinase will transform the mechanically disrupted tissue into a suspension of intact cells. In this regard, PG is the best pectolytic enzyme to be used for this purpose [10].

The phenomenon of hard-to-cook (HTC) legumes was found to be dependent upon the presence of water-insoluble pectic substances in the middle lamella that keep the firmenss of the legume tissues. HTC dry broad beans (*Vicia faba* L.) possess a hard middle lamella, and some early studies [58] showed that when they were soaked in a citrate buffer pH 4.5 containing 0.1% of a commercial pectolytic preparation (Rohament P) at 45°C for 12 hours before cooking, the enzyme did not affect the grain wetting coefficient. Moreover, large amounts of galacturonic acid and reducing sugars were leached out into the soaking medium. As a result, the cooking period was reduced and texture of cooked beans (assessed as Kramer's shear force) was severely soften by the presence of the enzyme preparation in the soaking water. Accordingly, it was speculated that the activity of the pectolytic preparation was acting upon insoluble protopectin, leaching out galacturonic acid and sugars in the soaking water, and leading to a loss of tissue cohesion [7, 58]. In this regard, results from later experiments on HTC broad beans soaked in an aqueous solution of a crude extract of pectinase activity produced by *A. sojae* (ATCC 20235) showed that the soaking time was reduced by half and the cooking time of beans was reduced to one sixth with respect to control experiments without the pectinase treatment. Results showed that the insoluble pectic substances (protopectin) of the middle lamella of the bean shell and cotyledons were degraded by the pectinase activity, causing the loss of tissue coherence and leading to a soft texture of the cooked food (**Figure 8**) [59].

10. Enzymes for grape juice and wine production

Grape (*Vitis vinifera*) production can be found on every continent except Antarctica because the vine plant is able to adapt to a wide range of environments, and because of the increasing importance of fermentation-based industries in the world economy. The grape berry has three major types of structures (**Figure 9**): pulp (80–85% of total weight), skin (7–11%), and seed (2–6%) [60], with the sheer bulk of juice being derived from the pulp, and remarkably, aromas and red grape juice colour is derived from the skin. Enological enzymes can be used at several stages in juice and wine processing and their application in the wine industry started in the 70s [61]. The main stages for winemaking are: a) pre-fermentation processing, b)

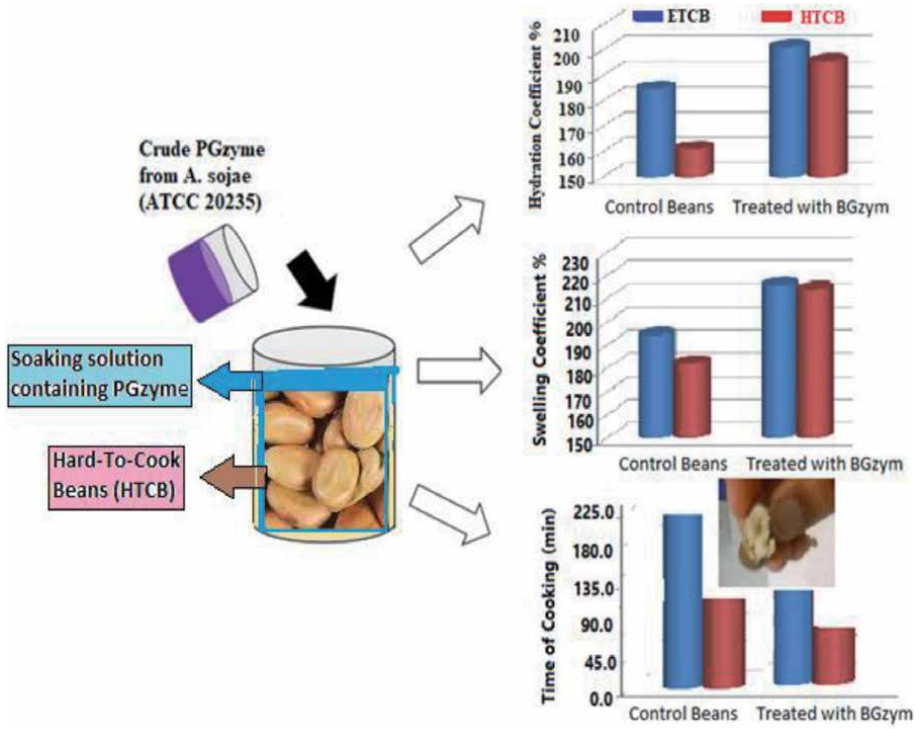

Figure 8.
Effect of 10 U PGzyme /ml at 40°C on the Hard-to-cook beans (HTCB) during soaking and cooking time [59].

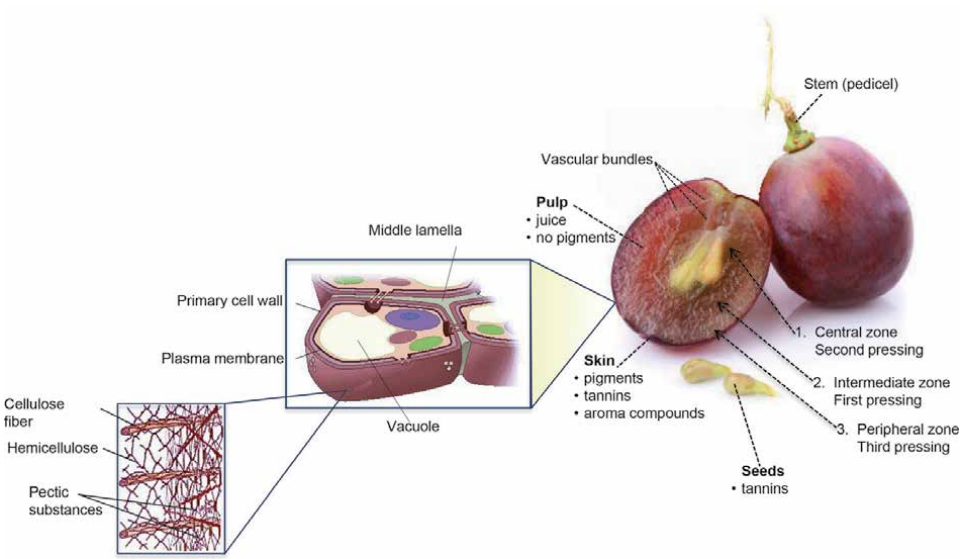

Figure 9.
Grape berry diagram and tissue organisation.

fermentation, c) post-fermentation processing, and ageing. Enzymes can be used in the first and third stages to improve: juice extraction, clarification, filtration, colloidal stability (by preventing haze from forming later in processing), aroma extraction, and in the case of red grapes, to improve colour extraction.

Commercial pectolytic enzyme preparations commercialised for grape juice and wine production are actually complex mixtures of several enzymes that degrade the cell walls and middle lamella of grape tissues, as well as polysaccharide colloids of the produced grape juices. The main enzyme activity of commercial preparations is polygalacturonase (*exo*- and *endo*-PG) accompanied with pectin lyase (PNL) [62]. In addition, these commercial preparations contain other enzymes that act in synergy with the former activities, among which, cellulases, hemicellulases, and other glycosidases (α-L-rhamnosidase, α-L-arabinofuranosidase, β-D-glucosidase) potentiate the lytic effect of these commercial enzyme preparations. When grape berries are infected by the mould *Botrytis cinerea* (grey rot or "pourriture grise") it is recommended to use specific pectolytic preparations that also contain *exo* β-(1 → 3) glucanases and β-(1 → 6) glucanases that degrade the β-D-glucan macromolecules produced by the mould and responsible for grape juice and wine spoilage [63]. Currently, commercial pectolytic enzyme preparations for enological use come from fungi belonging to the *Aspergillus* [37] and *Trichoderma* [61] genera that possess the status of GRAS organisms. Moreover, the International Organisation of Vine and Wine (OIV) requires manufacturers to inform their customers if the enzymes supplied have been produced from genetically modified organisms (GMOs). This information must be given either on the product label or on the technical documentation (resolution OIV-OENO 485–2012) and currently, both consumers and winemakers are not in favour of the GMO origin.

The endogenous pectolytic activities of grape cells and those of the wine fermenting yeasts [64] also participate in the whole lytic process of grape tissues. It is worth noting that only a low percentage (about 11.5%) of indigenous fungi strains associated with grapes and wine have been reported to be able to produce significant amounts of extracellular pectinases under enological conditions. Those strains belonged to *Aureobasidium pullulans, Filobasidium capsuligenum* [65] and to the yeast species included in **Table 2**. Under winemaking conditions, ethanol and sulphurous anhydride can be present at concentrations reaching 15% (vol/vol) and 120 mg L^{-1} respectively, which are quite restrictive conditions for grape and yeast pectinase activities [66] and to a lower extent, also for pectinase activities produced by filamentous fungi. These organisms have evolved to infect plant cells, they are well adapted to overcome the plant cell barriers and to degrade vegetable cell walls and pectic substances, as demonstrated by the number of pectinase encoding genes identified in fungi.

11. Grape processing and juice extraction

Destemming and crushing of grape berries are the first mandatory procedures for grape juice extraction, and simultaneously maceration starts as grape skins are torn into smaller pieces. At this stage, commercial pectolytic enzyme preparations can be used to degrade the polysaccharides of cell walls and middle lamella, thus facilitate juice release, liberation of polyphenols (pigments, tannins) and aroma molecular precursors that are located at the skin cells [67]. Pectolytic enzymes used in enology are selected preparations that are active at the acidic pH of grape juices (pH 3.0–4.0) under enological conditions and within wide limits of temperature [68], although at lower temperatures (15–5°C) the pectolytic activity decreases [37]. Pectolytic enzymes allow better extraction of the juice, but they also allow better extraction of the components of skins and occasionally of seeds. As shown in **Figure 9**, the pulp intermediate zone is easily released and crushed, whereas the peripheral zone, which includes skin cells, is the most difficult to extract and needs extra pressure to release its components. If grapes are not ripe enough, extraction of vegetal and astringent compounds from skins and seeds will depreciate the juice

quality. In this case, the fruit should be processed without the addition of pectolytic enzymes and avoiding maceration procedures, and consequently, the yield will considerably decrease, but the obtained juice will be low in bitterness and astringency. Thus, we see that enzyme addition at this stage should be wisely used to improve grape varietal characteristics and give character to the juice and wine. Normally, the more the damage is done to the skin and internal berry cell walls, the greater the release of berry components. Consequently, both aromas and flavours increase.

In the case of red-wine making, red grape skins are allowed to be present during the alcoholic fermentation and the polyphenols responsible for the red colour and sensory properties such as astringency or mouth-filling, which include anthocyanins and tannins among others, are further extracted as the ethanol produced during the fermentation facilitates their release from skin cells [69]. In the case of white and rosé wines, grape musts are submitted to pressing avoiding further contact with skins and seeds.

12. Grape juice clarification and filtration

In fact, white grape juice clarification was one of the first applications of enzymes in enology. After pressing, the grape juice is turbid as it contains numerous particles that confer a negative trait. Clarification can be achieved by gravity spontaneous setting, but in most occasions, it should be induced and facilitated by the addition of commercial pectolytic preparations [70, 71]. In all cases, the addition of pectolytic enzymes accelerates the clarification process; its technological efficacy is easily determined by pilot experiments measuring juice turbidity, and the methodology to be employed can be optimised for each specific type of elaboration. Further clarification after fermentation will facilitate the subsequent filtration of the resulting wine. Filtration is a key procedure to obtain a premium wine, and it is also most delicate because at this stage the colloidal matrix of the wine is very complex. The aim of adding pectolytic preparations at this stage is to increase wine filterability by degrading pectins and a variety of colloids (other polysaccharides, protein aggregates, glucans from *Botrytis cinerea*, yeast mannoproteins) responsible for clogging filtration membranes. Enzyme addition allows filtering larger wine volumes before filter clogging, and the enzyme cost is compensated for by the reduction in filtering costs and time reduction. Moreover, filtration is a process that all wines and most grape juices should undergo before bottling.

13. Extraction of polyphenols and aroma compounds: improvement of sensorial characteristics

The objective of adding commercial pectolytic preparations during red grape maceration stages is to improve the extraction of polyphenols and aroma molecules from the intracellular content of grape skin cells. The consequence of this higher yield of extraction is obtaining more structured wines, higher colour intensity, and wine aroma complexity [72]. Moreover, the resulting wine will be more easily clarified and filtered as well as the juice yield will be substantially increased. It is important to note that among the secondary activities present in commercial enological enzyme preparations, cinnamoyl esterase activities should be eliminated because they hydrolyse ester bonds of hydroxycinnamic acids, which are present in red grape skins [73], and release the free phenolic acids. These are substrates for the cinnamate decarboxylase of some *Saccharomyces cerevisiae* strains and of the wine spoiling yeast *Brettanomyces*. Cinnamate decarboxylase transforms the free phenolic

acids into volatile phenols that confer off-odours described as medicinal odour, leather or smoky to white wines, and barnyard or horsey odours to red wines [74]. Therefore, it should be made sure that no traces of cinnamoyl esterase activities are present in enzyme preparations for enological use. In addition, the pectin methylesterase activity (PE) should be negligible in the commercial enzyme preparations because no methanol should be generated when they are added [75].

The commercial enzyme preparations may also potentiate wine aroma expression by releasing volatile molecules that in their glycosylated form are not volatile, and consequently odourless. Primary or varietal aromas of wine are those whose origin are molecules of the grape cells, and they provide the characteristic aromatic profile of the grape variety used to elaborate the wine. Most of those volatile molecules appear in the grape cell as bound forms to sugars, becoming non-volatile and odourless. Glycosidic enzymes hydrolyse the linkage to the sugar residue and release the odorous molecule [76]. In addition, pectinolytic activities collaborate to break down cell walls and thus, liberate the aromatic volatile molecules.

It is important to point out that glycosidic enzymes used in enology should possess high specificity for their substrate, and β-glucosidases that hydrolyse anthocyanin glucosides should not be present in the commercial enzyme preparation because they provoke dreadful consequences of colour loss in red wines.

Finally, it should be underlined that enzymes can only extract what is already in the vegetable cells and tissues, and a good quality starting material is a requirement to obtain processed foods and beverages of premium quality. Pectinases can help to speed-up processes of extraction, maceration, clarification, filtration, and colloidal stabilisation of the processed product and thus they enhance product quality and improve the efficiency of the process, nevertheless when they are not properly used, they can spoil the final product. Additionally, pectinases can be used to upgrade food industry by-products to obtain value-added products and to produce animal feedstock. In this regard, pectinases have become a current trend in the feed industry and their use opens new perspectives for further research in the production of fungal pectinases.

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Chapter 5

The Microbial Degradation for Pectin

Abdelrahman Mosaad Khattab

Abstract

Pectin considers one of the most plentiful natural components having many applications. It is widely distributed in the middle lamella and cell walls of the terrestrial plant in various concentrations. Pectin is a heteropolysaccharide that involved galacturonic acid and methanol as the main components. Pectin is degraded by the pectinase enzyme, producing several compounds that have industrial applications. This Enzyme is produced by several organisms such as plants, protozoa, nematodes, insects, and microorganisms. However, the microbial source is the most common in commercial production due to its massive applications in various industries. Consequently, this chapter will show the importance of microorganisms to degrade pectin, the different types of microorganisms that can degrade pectin, and their applications.

Keywords: pectic substances, depolymerization, pectinase, application

1. Introduction

Pectin considers one of the most plentiful natural compounds having different applications. It is heteropolysaccharides that compose the main components of the middle lamella and primary cell wall of higher plants and are responsible for the cohesion and the structural integrity of plant tissues [1, 2]. Generally, rhamnogalacturonans and galacturonans are two main chemical components in pectic materials, where C-6 carbon of galactate is oxidized along with arabinogalactans and arabinans. The produced components are colloidal polymeric in nature and structurally heterogeneous, containing a large backbone of anhydrogalacturonic acid units. The carboxyl groups of GalA are partially esterified by methyl residues and totally or moderately neutralized by different ions as potassium, sodium, and ammonium. α-1,4-d-galacturonate units are working as a master and connecting about 2-4% of L-rhamnose units that linked [β- (1 → 2) and β-(1 → 4) to galacturonate units. The side chains include simple carbohydrates (galactan, arabinan, arabinogalactan, fucose, or xylose) but vary in length and composition. They are associated with the central chain through their C1 and C2 atoms [3]. Hence, the main chain of the pectin polymer consists of galacturonic acid (GalA) linked by α-D-1,4- bonds and form three backbone regions [4], **Figure 1**:

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1. SMOOTH REGION OR HOMOGALACTURONAN (HG), represents about 65% of pectin [6] and involves long stretches of (1 → 4)-linked d-galactopyranosyl uronic acid residues [7]. Further modifiable occurs by the process of Methyl esterification at C-6, or acetyl groups at C-2 and C-3 position [8].

2. HAIRY REGION OR RHAMNOGALACTURONAN (RG I) the branched area, represents 20-35% of pectin, involves the side chain of the repeating disaccharide unit [α- 1, 2-rahmnopyranose residues] linked by (1 → 4) disaccharide [9]. Sometimes, lateral chains contain glucuronic acids and fucose found mostly to create the structure more complex.

   - This region is acylated and frequently substituted with arabinans, galactans, and arabinogalactans linked to rhamnose residue [10]. Also, xylose is found for substitutions [11].

   - RGII region, although the name, is not structurally related to RGI. It is a branched pectic domain-containing HG backbone substituted with heteropolymeric side chains involving different sugars [12]. Containing side chain of D-Apiose, 2-Omethyl-D-xylose, and 2-O-methyl-L-Fructose. The galacturonic residues are usually acetylated at the C-2 or C-3 position in rhamnogalacturonan 1 [13].

Most molecules are formed in the series by D-Galacturonic residue. Polymers do not make up a straight string in an aqueous medium, but it is curved and extended with high flexibility. The contrast was found in the configuration of pectin from different sources, and the pectin properties are strongly related to the

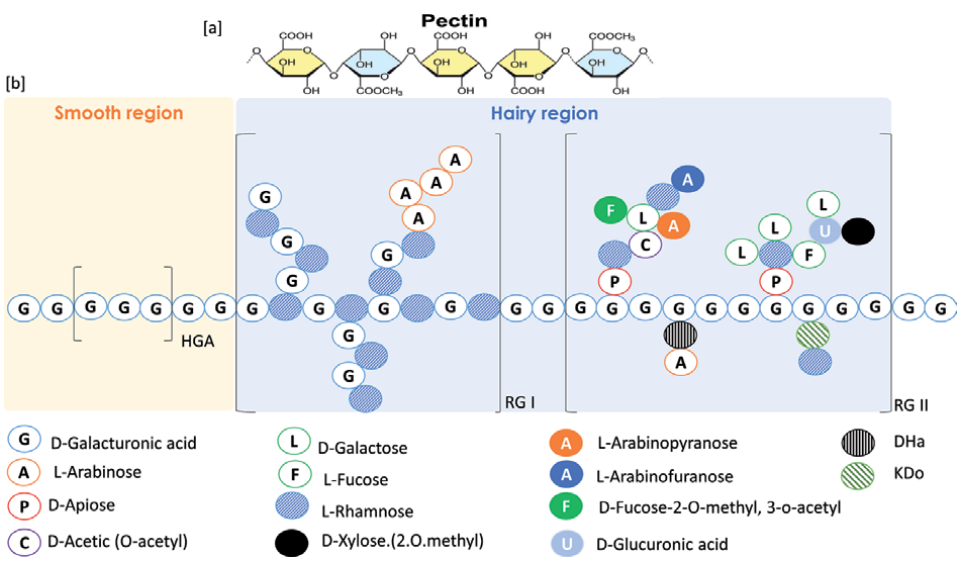

Figure 1.
(a) pectin Structure (b) Pectin structure in traditional model displaying the homogalacturonan (HG) as the backbone, smooth region, and 60-sugar residue. Feruloyl pectic acids are engaged in organizing cell expansion, resistance to diseases, and lignification initiation. The properties of the pectin the hairy region consisting of Rhamnogalacturonan-I (RG-I) or Rhamnogalacturonan-II (RG-II) units [inspired by Noreen et al., [5].

methylation of galact-uronic acids residues, which is usually 70% [14]. The acidic and neutral pectin has ferulic acid on non-reduced ends of neutralarabinose and or galactose including domains. Pectin carries about one teruloyl residue for every matrix is complicated due to the interaction of the domain structure of pectin between themselves and with other ionized inorganic and organic compounds [15].

The American chemical community, ranked according to the nature of the molecular arrangements of pectic substances into four main groups [16]:

a. PROTOPECTIN is considering the pectin parent were on the restricted hydrolysis yields pectinic acid or pectin [17]. And located in the middle lamella, serving as the glue to hold cells together in the cell walls. Also, it is water-insoluble due to its large molecular weight, formation of ester-bond between carboxylic acid groups (in pectin) and hydroxyl group (in other constituents of the cell wall), and salt bonding between the carboxyl groups (in pectic substances) and basic groups (in proteins).

b. PECTIC ACID or pectate: is a polymer of galacturonan containing a few methoxy groups and is water-soluble.

c. PECTINIC ACID is a polymer of galacturonan containing a significant amount of methoxy groups (up to 75%). Under a suitable condition, can forming gel with sugar and acid.

d. PECTIN (polymethyl galacturonate) is a soluble polymeric material in which almost the carboxyl group of galacturonate units (about 75%) are esterified with a methyl group. Pectin as pectinic acid can be forming a gel with sugar and acid under favorable conditions [18].

2. The depolymerization of the pectin polymer into a simpler form

The pectin depolymerization occurred either physical, chemical, or enzymatic methods, **Figure 2**.

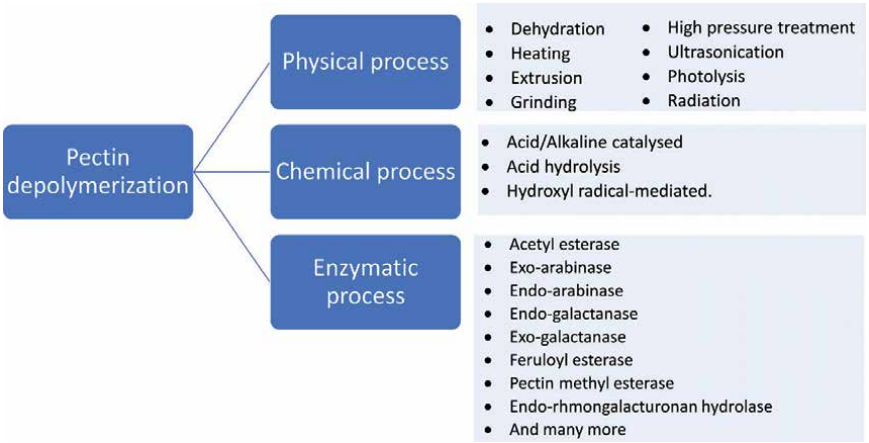

Figure 2.
The different depolymerization processes of various types of pectin [inspired from Satapathy et al., 2020 [19]].

2.1 Physical process

Includes high-pressure treatment, ultrasonication, radiation, and photolysis. At a pH value lower or higher than 3.5, the acetyl, methoxyl, and neutral sugar groups are eliminated, and the polymer backbone is cleaved.

2.2 Chemical process

Acid or base hydrolysis can catalyze the splitting of chains by the β-elimination reaction. The dissolution takes place at a glycosidic linkage next to an esterified **GalA**. As a result of the higher methoxylation degree (DM) of pectin becomes extra susceptible to base-catalyzed reactions rather than a low DM pectin. However, by acid hydrolysis (pH < 3.0), pectin hydrolyses with low DM are faster in comparison to pectin with high DM [20].

2.3 Enzymatic process

Wide range of enzymes used for the polymer degradation allowing region-selective depolymerization under mild conditions. So that, using enzymic degradation gain the interest of many researchers.

3. The pectinolytic enzymes

More than a century ago, DeBary at1886 showed pectinase's importance as a virulence factor in the decomposition of pectin in the plant cell wall [21]. Then, these enzymes have been used for food processing at the domestic level at first, to use in the industrial sector in 1930. Till1960s, pectinases were practically used in fruit juice and wine for clarification [22]. Nowadays, pectinases have received attention worldwide as an eco-friendly biocatalyst that contains a 25% share in the global enzyme market for food and beverages [23, 24]. In 2016, the pectinases market reached 30.0 million $ and was estimated to rise to 35.5 million $ by 2021 [25]. Pectinolytic enzymes or pectinase enzymes are a group of complex enzymes that catalyze the degradation of pectin-containing substances. These enzymes account for 10% of the produced global industrial enzymes [1] and are secreted in plants, insects, Nematoda, protozoa, fungi, yeast, and bacteria [22]. Although plant and microorganisms are the major sources for pectinase enzyme generation, microbial sources (Fungi, yeast, and bacteria) have been selected to be the primary ones due to technical and commercial viability [26].

Pectinolytic enzymes have been classified according to three criteria [27, 28]:

- The used substrate (Pectin, pectic acid, or oligo GalA)
- The cleavage type (trans elimination or hydrolysis)
- The action mode (random cleavage-depolymerizing or endoliquifying enzymes, or end-wise cleavage endo or exo saccharifying enzymes.

For most enzymes, the type of cleavage is random (Endo) or terminal (Exo). The recent classification based on the action mode on pectin, preferred substrates, and products, **Table 1**, where these enzymes are classified into three types [30]:

E.C. suggested name	Common name	E.C. no.	Mode of action and cleavage	Catalytic reaction
PROTOPECTINASES			Cleavage of non-methylated molecules of galacturonate unit, hydrolyzing the glycosidic bond	Protopectin $+H_2O \rightarrow$ Soluble pectin
ESTERASE ENZYMES				
a) Methyl esterase	Pectin esterase	3.1.1.11	Random cleavage of the methyl ester group of galacturonate unit	Pectin $+nH_2O \rightarrow$ Pectate+ Methanol
b) Acetyl esterase	Pectin esterase	3.1.1.11		Pectin $+nH_2O \rightarrow$ Pectate+ n Acetate
DE-POLYMERASE				
a) Hydrolases				
I) Polygalacturonase (PG)			- Catalyze the hydrolytic cleavage of α-1, 4-glycosidic linkage in pectic acid	
- Exo-PG	Pecate polymerase	3.2.1.67	Terminal cleavage from the non-reducing end of the polygalacturonic acid	Pectic acid$+H_2O \rightarrow$ Mono-galacturonates
- Endo-PG	Pecate polymerase	3.2.1.15	Random cleavage of pectic acid	Pectic acid $+H_2O \rightarrow$ Oligo-galacuronates
II) Polymethylgalacturonase (PMG)			- Catalyze the hydrolytic cleavage of α-1,4-glycosidic linkage in pectin	
- Exo-PMG	Pectin polymerase		Terminal cleavage from the non- reducing end of pectin	Pectin $+H_2O \rightarrow$ Methyl-mono-galacturonates
- Endo-PMG	Pectin polymerase		Random cleavage	Pectin $+H_2O \rightarrow$ Oligo-methyl-galacturonates
b) Lyases				
I) Polygalacturonate Lyase (PGL)			- Catalyze the cleavage of α-1,4-glycosidic linkage in pectic acid by trans-elimination forming unsaturated galacturonates.	
- Exo-PGL	Pectate lyase	4.2.2.9	Cleavage of penultimate bonds from non-reducing end	Pectic acid → Unsaturated digalacturonates
- Endo-PGL	Pectate lyase	4.2.2.2	Random cleavage	Pectic acid → Unsaturated oligo-galacturonates

E.C. suggested name	Common name	E.C. no.	Mode of action and cleavage	Catalytic reaction
- Oligogalacturonate lyase	Pectate lyase	4.2.2.6	Terminal cleavage	Pectic acid → Unsaturated mono-galacturonate
II) Polymethgalacturonate lyase (PMGL)	Catalyzes cleavage of α-1,4-glycosidic linkage in pectin by trans-elimination forming unsaturated methyl galacturonates at the non-reducing end.			
- Exo-PMGL	Pectin lyase		Terminal cleavage	Pectin→ Unsaturated methyl mono-galacturonates
- Endo-PMGL	Pectin lyase	4.2.2.10	Random cleavage	Pectin→ Unsaturated methyl oligo-galacturonates

Table 1.
Classification of pectinases enzymes [inspired from Parissa et al., 2011 [29]].

3.1 Protopectinase

Protopectinase, which catalyzes the solubilization of protopectin in the presence of water to release soluble pectin. The catalyzation process could occur through the reaction at sites having three or more non-methylated molecules of GalA, hydrolyzing the glycosidic bond [31]. According to the catalytic action, these enzymes are classified into two types:

1. Type-A reacts with the smooth region or inner site of insoluble protopectin. It is reported that, in the culture filtrate of many microbes as *Trichosporon penicillatum* SNO 3 (S-type), *Galactomyces reessi (L-type)*, and *Kluyveromyces fragilis* IFO 0288 (F-type). F, S, and L types have the same molecular weight of about 30 kDa and are more optimum at pH 5.0. in addition, only F-type is an acidic protein, while others are basic in properties. These enzymes are responsible for a decrease in the viscosity with an enhanced rate of reduction for polygalacturonic acid in the reaction medium. Similarly, *Bacillus subtilis* IFO 3134 produced R and N-type with 35 and 43 kDa protein, respectively, and highly active at pH 8.0 and temperature 60 °C). These enzymes can help in the reaction of trans-elimination by breaking the glycosidic linkages at the protopectin [32] and categorized according to the pattern of action as endo (random) or exoenzymes (terminal).

2. Type B is responding at the site of polysaccharide chains or outside of the protopectin. It links the chain of polyglacturonic acid and the components of the cell wall [33]. It is isolated from Trametes sunginea (T-Type) and *B. subtilis* IFO 3134 (Ctype), having different molecular weights 55 and 30 kDa, respectively, and several isoelectric points (T: 8.1 and C: 9.0). The kind of these enzymes is abundantly found in agro-products as orange, lemon, hassaku, carrot, apple, burdock, radish, and sugar beet, acting on protopectin particularly [34].

3.2 Esterase

Esterase, which removes methoxyl and acetyl esters from pectin forming polygalacturonic acid. Where catalyzes the de-esterification of pectins. These enzymes from fungal originate work by arbitrarily eliminating the methyl groups via a multi-chain mechanism, while from plant source work by attacking either the next terminal to a free carboxyl group or the non-reducing-end and progressing linearly through a single-chain mechanism [35]. Esterase enzymes are producing mainly from microorganisms and have a negligible effect on the viscosity of a pectin solution without divalent cation as barium (Ba^{2+}), calcium (Ca^{2+}), and strontium (Sr^{2+}). However, it is reported that the presence of Ca^{2+} ions releases a maximum effect at either small or large preparations [36].

Some of the purified esterase acting against the reducing end. While others are targeting the non-reducing-end of pectin. The molecular weight for these enzymes ranges from 22 to 90 kDa, referring to the difference of the protein confirmations. For the work efficiency of esterase, various ranges of pH (5-11) and temperature (40 – 70°C) should be applied. However, the product optimization of the fungal esterase has a lower pH value versus the bacterial one [37]. Depending on the functional group target, pectinesterase is classified into:

1. *Methyl-esterase*, which divides the methyl ester group of pectin. Freeing methanol and converting pectin into pectic acid or pectate via a single-chain mechanism. The chain length of pectin polymer is not reduced [38]. Various

isoenzymes of pectin methylesterases are isolated and characterized from different sources, considering the functional group's target [39]. Two types of pectin methylesterase A (PmeA) and B (PmeB), were isolated from *Erwinia chrysanthemi* [40] and *E. chrysanthemi* 3937 [41] and were well-studied. The PmeA acting functionally to be extracellular, while the PmeB enzyme acting on the outer membrane.

2. *Acetyl-esterase*, which catalyzes the hydrolysis of acetyl ester residues of pectin, forms acetate in pectic acid [42].

3.3 Depolymerase

Depolymerase, a range of depolymerizing enzymes degrade the pectic substance through cleaving of α-(1 → 4)-glycosidic bonds in DGalA units either by trans elimination or hydrolysis [43]. Split the -(1,4)-glycosidic bonds in pectins either by hydrolysis (polygalacturonase) or by transelimination (lyases).

1. *Hydrolases* include polygalacturonase (PG) and polymethylgalacturonase (PMG).

   i. Polygalacturonases (PG), that split the glycosidic linkage in the presence of water molecules across the oxygen bridge. Forming a D-galacturonic acid monomer. The structure confirmation of these enzymes loses when it reacts with pectin, which may occur due to the presence of free carboxylic groups in the target molecules. The viscosity of the interaction solution reduces with an increase of reducing end-groups. PG is the most enzyme studied and industrially applied because of its depolymerization specificity via the hydrolysis process [44, 45]. Depending on the pattern of action, PG is categorized into:

   - Exo-PG, which targets the terminal groups of the pectic molecule, lowering of chain length gradually.

   - Endo-PG, that attacks all chain links arbitrarily, resulting in more incisive and faster consequences.

However, rhamnopolygalacturonase catalyzes cleavage within or at the non-reducing terminals of the rhamnogalacturonan core chains [46, 47]. Various microorganisms can produce PG with several biochemical properties and modes of action. Most PGs stimulate the hydrolysis rate at an optimum temperature ranging from 30 to 50°C with ideal pH that ranges from 3.5 to 5.5. It is reported that almost both exo-PG and endo-PG are synthesized in acidic conditions. While some exo-PG are produced at high basic pH (11.0) by certain bacterial and fungal species as *Bacillus* sp. KSM-P410, *Bacillus licheniformis*, and *Fusarium oxysporum* [48]. Whereas rhamno-PG is more efficient and stable at pH 4.0 and temperature 50°C. The molecular weight average for exo-PG and endo-PG is 38 – 65 kDa, while rhamno-PG is 66 kDa [49].

   ii. Polymethylgalacturonase (PMG) can catalyze the hydrolytic cleavage of α-1,4-glycosidic linkage in pectin. It is divided according to the action pattern into:

   - Exo-PMG, that targets the terminal groups of the non-reducing end of pectin, releasing methyl mono-galacturonate.

- Endo-PMG, that attacks all chain links randomly. Resulting in more incisive and faster consequences of oligomethyl-galacturonates.

Lyases (trans eliminases), in which trans-eliminative breakdown for pectinate polymers or pectate through catalyze the Polygalacturonate depolymerization and pectin esterification, by splitting the C-4 of the glycosidic linkage followed by hydrogen removal from the C-5 releasing an unsaturated product with the unsaturated bond between C-4 and C-5. For activation, some cytoplasmic or intracellular lyases, need ions as Ni^{2+}, Co^{2+}, and Mn^{2+} [50].

According to the acted substrate, lyase is divided into two types [51]:

i. Polygalacturonate lyase (PGL) that requires Ca2+ ions for its activation. Used mainly in baby food products [52]. That classified into:

   - Exo-PGL, target the non-reducing terminal of pectic acid, releasing unsaturated di-galacturonates.

   - Endo-PGL, which works in an unsystematic cleavage fashion on the substrate, producing unsaturated oligogalacturonates.

   - Oligo-D-galactosiduronate lyase, which acts on the terminal position of unsaturated di-galacturonate, released initially by the pectate lyase action, forming mono-galacturonates [53].

ii. Polymethylgalacturonate **lyase** (PMGL), which does not need any metal ions for their activation, although arginine (Arg 236) residues are found at the position of Ca2+ ion as observed in the pectate lyases [54]. Which categorized into:

   - Exo-PMGL, that degrades pectin through stepwise transeliminative cleavage, releasing unsaturated methylmonogalacturonates [55].

   - Endo-PMGL, which acts randomly on the pectin by cleaving α-1,4-glycosidic linkages, producing unsaturated methyloligogalacturonates.

Overall, pectin lyases originated mainly from microorganisms, which lead to change in the biochemical properties according to each microbe. These enzymes working efficiently in the temperature range 40-50°C, and alkaline pH 7.5-10.0. The molecular weight of lyases is ranging from 22-90 kDa, while PMGL reached 89 kDa from *Aureobasidium pullulans* LV-10 and 90 kDa from *Pichia pinus*. Whereas the molecular weight for PGL of 55 and 74 kDa was reported in *Yersinia enterocolitica,* and *Bacteroides thetaiotaomicron*, respectively. The point of isoelectric for some lyases are ranged from 5.2 to 10.7. While others are still unexplored [56]. Many enzymes act in the adjacent chains of RGI and RGII as exogalactanase, endogalactanase, α- and β-galactosidase, α-L-arabinofuranosidase, exoarabinase, and endoarabinase [57].

4. The microbial producers for pectinases

Pectinolytic enzymes are produced by various plants and microorganisms, while animal cells can't [58]. Several strains of fungi, yeast, and bacteria are producing different types of pectinolytic enzymes. While the fungal sources provide the largest variety of bulk commercial enzymes and have a broad diversity of applications, well documented by GA [59].

4.1 Fungi

Three fungal classes get the most attention in SSF:

1. Phycomycetes, as genera of Mucor,

2. Ascomycetes, as genera Aspergillus,

3. Basidiomycetes, as genera white-rot fungi [60].

Many fungal strains can produce various types of pectinase enzymes. The produced metabolites by *Aspergillus niger* are safely used and involved in GRAS (Generally Regarded As Safe), so that it is the most broadly strain used in industries [61]. *A. niger* can produce various pectinases involving esterase, PGL, and PMGL. Currently, the enzymes of *A. niger* are used in wine industries and fruit juice [62]. It is reported that almost all the fungi pectinases have acidic nature, so it is applicable to work only in acidic conditions [63]. However, the pectinase production by *Aspergillus* strains produced higher via SSF than in SmF [64]. Wherever the most commercial pectinases are produced from fungal sources in the industry by *Trichoderma* and *Aspergillus* [65].

4.2 Yeast

In 1951, Luh and phaff produced for the first time endoPG from yeast [66]. They identified four species of pectolytic yeasts: *Saccharomyces fragilis, Torulopsis kefir, Candida pseudotropicalis* (all later renamed as *Kluyveromyces marxianus*), and *Saccharomyces thermantitonum* (reclassified as *Saccharomyces cerevisiae*) [67]. Pectolytic yeasts can be producing PG, lyase, or esterase, depending on the pH, temperature conditions, and substrate availability. For example, *Candida*, *Saccharomyces, and Kluyveromyces* can be producing PG (mainly endo-Polygalacturonase), whereas *Rhodotorula* can be releasing both pectin esterase and PG [68].

S. cerevisiae is considered the most experienced yeast. Initially, it had believed that *S. cerevisiae* is free of pectolytic enzymes [69]. However, some strains have been shown since then reduction ability for pectin [70]. PG Activity is the main pectolytic activity in *S. cerevisiae* and was reported in several strains [71]. It is noted that most pectolytic activities have been described in the indigenous yeasts [72]. These yeasts have been mainly discovered during different fermentation [73] or through the clarification and pressing of concentrated juices [74].

4.3 Bacteria

Chawanit Sittidilokratna *et al*. [75] performed screening of pectinase-producing bacteria and the effectiveness evaluation for bio-pulping. At first, Pectinolytic bacteria were screened from six identified and 118 unknown isolates. Twelve strains gave positive results, including three of *Erwinia carotovora subsp*. *carotovora*, two of *Erwinia chrysanthemi* and seven of *Bacillus* sp. Crude pectinases were prepared from the selected strains. Then, investigate the activity of three types of pectinase (PG, pectate lyase, and pectin lyase). The results showed the highest PG production from *Bacillus* sp. strain N10 and *E. chrysanthemi* strain N05. N10 was isolated from paper mulberry bark, while N05 was isolated from onion [76]. *Bacillus licheniformis* KIBGE-IB21 was isolated from rotten vegetables and produce pectinase at certain

conditions [77]. However, the commonly used bacteria for pectinase production are *Aeromonas caviae, B. licheniformis, and Lactobacillus* [32].

5. Applications of pectinase

Over the years, pectinases have been used in several applications as plant fiber processing, textile, coffee, and tea fermentation, industrial wastewater treatment, oil extraction, wine-making, wood preservation, pectic pre-treatment, degumming and retting of fibers, protoplast formation [78], and fruit industry [79]. With increased knowledge and understanding of the mechanism of pectin-degrading enzymes, pectinases have made their way into other biotechnological processes as purification of plant viruses [80] and paper and pulp making [81]. Today, pectinases represent almost 5% of global enzyme sales with approaches 35 million dollars of the industrial market [82].

The biotechnology application for these enzymes has expanded in recent years. These occurred especially in food and related industries, to increase the product quality, product stabilization, increase the efficiency of extractive processing, improve the flavor and by-product utilization [83], **Figure 3**.

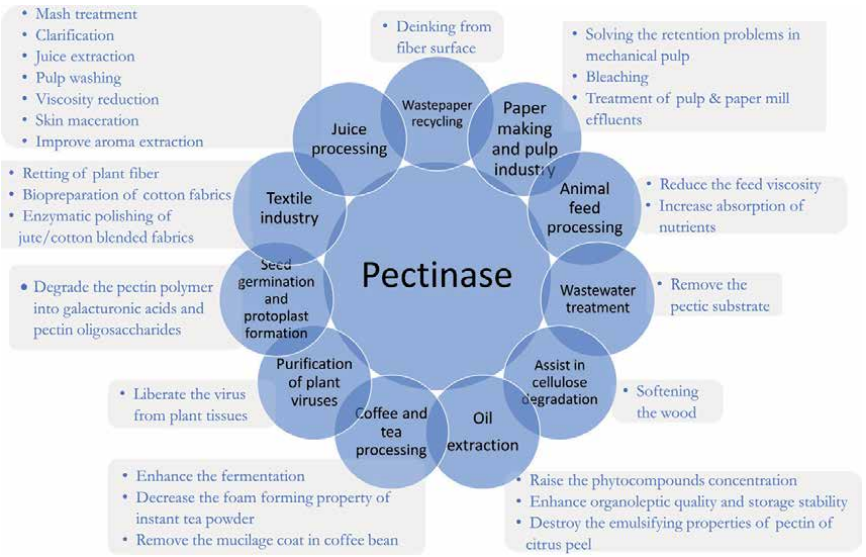

Figure 3.
Applications of pectinases in the various biotechnological and industrial sectors with the potential purpose [inspired by Garg et al., 2016 [84]].

5.1 Juice processing

Pectinases use in the fruit juice and wine industry since the 1930s [85]. That occurred for reduction maceration and viscosity which contribute to increasing the obtained press juice to the de-peel pulps. Similarly, reducing the viscosity of fruit drinks [86]. Fogarty and Kelly stated that pectinases use in wine clarification [87]. The juice industries produced commercially three different types of juices:

1. Sparking clear juices,

2. cloudy juices, and

3. unicellular product.

So, the objective aim of using pectinase in the three types is different, **Table 2**.

5.2 Wine processing

To clarify wine, the enzymes added before fermentation of white wine musts. Musts are made from pressed juice without any skin contact to rapid clarification. Thermovinification technology, during the grape mash heating for few hours releasing large amounts of pectin. So that, adding pectinases is necessary to the heated mash that leading to reduce the juice viscosity. Adding pectolytic enzymes enhances the color, which probably refers to the breakdown of the cell structure, which allows escaping more readily pigments (anthocyanins) [92].

The treated wines enzymatically showed more stability with lowering the filtration time comparing with the untreated wines [93]. The enzymatic treatment raises the levels of alcohol production in fermented grape must, observing an increase in 2-phenyl ethanol and iso-amyl alcohol and a decrease in concentrations of n-propanol [94]. Servili *et al*. [95] stated that adding pectinases in the wine-making process leads to raising the levels of methanol in wine due to the pectinesterase activity. The methanol concentrations should be regulated due to the toxicity of methanol, therefore in a commercial mixture of pectin esterase should be at lower concentrations [96].

Reddy and Reddy [97] reported that the combined effect of both fermentation by yeast cultures and pectinases treatment on Alcohol production. Consequently, the yield of juice increase when treated with 0.6% of enzyme concentration and conduct the fermentation at Ph 4.5 and 30°C for 12 h.

The most functions of pectinolytic proteins within the wine-making preparation are to bolster the extraction process, maximize juice surrender, encourage filtration and escalating the enhance and color [98]. Enzymatically treated wines appeared more soundness with decreased filtration time in comparison to control wines [99].

The juice industries produced	**The aim of using pectinase**	**Examples**
Sparking clear juices	• To increase the obtained yield during the pressing	• Manufacture of apple juice as natural, unclarified, unfiltered, and pulp-containing juice
	• To straining of the juices	
	• To remove the suspended particles	
	In apple: *mostly used*	• The production of French cider apples [89]
	• To depolymerize the highly esterified pectin [88]	
	In cider: enhances the good fermentation to produce high-quality juices and aromatic cider	
Cloudy juices	• To stabilize the cloud of citrus juices, purees, and nectar through the high levels of PG that have been involved, achieving high carotene and dry matter content of the product	Pectinex Ultra Sp-L is a commercial enzyme used specifically for the preparation of carrot puree [90].
Unicellular product	To transform the organized tissue into suspension of intact cells through maceration process, for nectar and pulpy juices [91]	Such as baby foods, yogurt, and puddings

Table 2.
The objective aim of using pectinase in the industrially produced juice with examples.

Treatment of macerated natural products with pectinolytic chemicals, sometimes recently the expansion of inoculum come about in progressed characteristics of wine [100]. Clarification of must earlier to the onset of alcoholic aging to moves forward the tactile properties of white wine [101]. Bosso [94] detailed the higher levels of liquor generation in matured grape must be pretreated with pectolytic proteins and watched increment in iso-amyl liquor and 2-phenyl ethanol, while a diminish in n-propanol concentrations, Reddy and Reddy [102].

5.3 Textile industry

To treat the natural fibers such as ramie fibers and linen, sometimes pectinases are used to remove the gum before starting the textile making [103]. The old-age practice of retting by textile fibers as hemp, flax, and jute are prepared with the pectinases of certain microorganisms [104]. The traditional methods are using caustic alkaline solution (3-6% aqueous sodium hydroxide) at high temperatures to fulfill the consistent dyeing and finishing. This process can degrade the cotton fiber, needs large amounts of water for rinsing after completing the process, and high energy, releasing a toxic waste product that can damage the environment. For that, use a combination of pectinases and xylanase ensure the low discharge of chemical waste in the environment, producing lower odor, and improving the safety of textile workers with the fabric quality.

To remove the non-cellulosic impurities as pectin, protein, and fats from fiber using pectinase through a novel process called bioscouring. This process is conservation the energy, eco-friendly, and significant results in fiber damage without effects on the cellulose backbone [105].

5.4 Develop seed germination and protoplast formation

Das and Baruah mentioned that *Trichoderma reesei* isolated from areca nut husk released high polygalacturonase. The areca nut germination was better when treated with cell-free preparation of polygalacturonase enzyme than with distilled water [106].

For protoplast formation of the plant cell, enzymatic and mechanical methods have been used. In the mechanical method, the protoplast formation involved separating plasmolyzed tissue with a sharp-edge knife, releasing the protoplast through de-plasmolysis. The mechanical method is limited with the low yield of the protoplast. Hence, the enzymatic method is mostly used for this purpose. A combination of both pectinases and cellulases has been used for the protoplasts formation from every plant tissue that has not lignin [107]. Pectin-oligosaccharides (POS) have different biological activates involving plant growth promotion and antimicrobial agents. POS is not digestible by humans, where fermented by microbial flora as *lactobacillus species* and *bifidobacteria sp.*, stimulating their growth. POS is released by chemical and enzymatic degradation of pectic substrates. In enzymatic degradation, pectinase has been applied to break down the $\alpha -1, 4$ glycosidic linkages of pectin polymer to galacturonic acids and pectin oligosaccharides [108]. Immobilized pectinase has been used for controlled enzymatic reaction with specific characterization and defined range of polymerization degree, avoiding producing large amounts of monosaccharides in batch production [109].

5.5 purification of plant viruses

The highly pure preparation of viruses is necessary for studying their physical, chemical, and biological properties. Various methods of purification can be selected according to the virus type. Pectinase's enzymes can be used to liberate the virus from the phloem tissues [110].

5.6 Coffee and tea processing

Marcia Soares *et al*. reported that pectolytic enzymes were used to hasten the removal of the jelly that surrounds the coffee cherry in the processing of green coffee beans, increasing the inferior quality of coffee beans [111]. Using pectinases develops the coffee quality also through converting the mucilage into sugars [112]. Similarly, pectinases treatment enhances the fermentation of tea by breaking down the pectin found in the cell walls of tea leaves, also destroying the foam-forming of the powder tea. It is reported that the used pectinases in tea process from alkaline fungal pectinase type [113].

So, the enzyme enhances the development of color, aroma total soluble solids, dry matter content, and active ingredients as theaflavin, thearubigin, caffeine, and high polymerized substances [114]. Masoud and Jespersen used PG in the fermentation of *Coffea arabica* produced by *Pichia* [115]. Adding these strains as started cultures in the coffee fermentation process help in mucilage degradation and the biological control of ochratoxin A which producing by *Aspergillus ochraceus* during the fermentation [116].

5.7 Oil extraction

Pectinases are applied in disrupting gels to assist the recovery of oils [117]. *Vegetable oils* of sunflower, olive, palm, coconut, or canola are obtained by extraction with organic solvents as hexane, a potential carcinogen [118]. With pectinase from alkaline type is preferable, lets the extraction of vegetable oils in an aqueous process through cell wall components degradation. Nowadays, the utilize of enzyme preparations involving hemicellulases, cellulases, and pectinase has raised the concentration of phyto-compounds as an antioxidant, polyphenols, essential proteins, lipophilic compounds, and vitamin E content, enhancing its organoleptic quality and storage stability [119, 120]. In 1992, Servili *et al*. stated that using an endo-PG extract from *Cryptococcus albidus* improved the olive oil extraction by adding the enzyme during the grinding process of olive [121]. Moreover, citrus oil such as lemon oil can be extracted by pectinases that destroy the emulsifying properties of the pectin of citrus peel [122]. Pharmaceutically, pectinases can improve the oil production from the medicinal plants to use as a treatment for various diseases involving depression, cancer, anxiety, microbial infectious ailments, and wound healing. However, pectinases will be contributing to the cosmetic and perfume industries. Using organic solvent in the extraction process might damage some critical functional groups. So, use pectinases during the extraction process will avoid that by destroying the emulsifying properties of pectin and promote the liquefaction of the cell wall components, releasing a better volume of products [123].

5.8 Assist in cellulose degradation

Pectinase plays a critical role by raising the access of cellulases to their substrates [124]. Spagnulo *et al*. [125] and Wang and Chang [126] stated that pectinase became the most important enzyme, since hydrolyzing the pectic surface of the lignocellulosic materials. The degradation of cellulose and hemicellulose was favored by the respective enzymes. Thus, pectic enzymes treatment is used for softening the wood-producing commercial softwoods [127].

5.9 Animal feed processing

Using various enzymes in the animal and poultry feed started in the 1980s with adding β-glucanase into barley and then wheat. After that, the xylanase enzyme was tested and achieve the best action in this case. Usually, the preparation of feed enzymes

is a cocktail of multi-enzymes containing proteinases, amylases, pectinases, xylanases, and glucanases. Adding enzymes to the animal feed is reduces viscosity, increases nutrient absorption, liberates the blocked nutrients by this fiber, and reduces the feaces amount [128]. Spraying feed with enzymes just before providing the feed, increases the food flexibility management, improving the feed digestibility through various mechanisms as direct hydrolysis, palatability improvements, changes in gut viscosity [129]. Consequently, enzymes supplementation to the animal feed improves significantly the digestive process, weight gain, feed conversion, and digestible energy intake [130].

5.10 Papermaking and pulp industry

With the biotechnology advancement increased dependence of pulp and paper industries. Many enzymes are used almost in papermaking for bio-bleaching and papermaking, such as pectinases, mannanase, and α- galactosidase [131]. Pectinase can degrade the galacturonic acid polymer, lowering the cationic demand of pectin solutions and the filtrate from peroxide bleaching of thermomechanical pulp [132]. Bio-bleaching of eucalyptus kraft pulp obtained by a mixture of alkaline pectinase and xylanases from Streptomyces sp. QG-11–3 [133], *Bacillus subtilis, Bacillus pumilus* [134], and *S. cerevisiae* [135]. Bio-bleaching results in less requirement of chemical-bleaching, giving the same pulp brightness, enhance the physical properties of the paper sheet, and reduce the organochlorine compounds in the effluent. Liu *et al.*, lowering the used pectin concentration in bio-bleaching by using cross-linked chitosan beads, leading to a sharp decrease in pectin molecular weight and cationic demand of pectin solution [136].

5.11 Wastepaper recycling

The major problem in wastepaper recycling is deinking process that needs a large amount of environmentally damaging chemicals. Bio-deinking using enzymes is less polluting, gives better quality, and is energy-saving. Pectinases, cellulases, hemicellulases, and ligninolytic enzymes are used for bio-deinking. These enzymes alter bonds near the ink particle, removing the ink from the fiber surface. Then, the resulting ink is removed by washing or floatation [137, 138]. A combination of pectinase and xylanase has been used for bio-deinking of school wastepaper [139]. Hence, bio-deinking lowers the values of biological and chemical oxygen demand in the effluent, reducing the treatment cost for wastewater to be environment friendly [140].

5.12 Wastewater treatment

The wastewater of the vegetable food industries contains pectic substrates. The typical treatment of its wastewater involves multiple steps that are high in cost, have longer times, and pollutes the environment [141]. Thus, using alkaline pectinases to remove the pectic substrate is a good alternative, cost-effective, and eco-friendly method, easing the decomposition by activated sludge treatment [142, 143].

6. Conclusions

The microbial degradation of pectin through various enzymes gains the interest of many researchers. In this chapter, microbial degradation for pectin has created an irreversible renaissance in the current era of innovation and the latest research to find the new utility of microorganisms and their products, a pure fact. This chapter showed the pectin structure generally with its different forms. Then,

several methods have been utilized in the pectin depolymerization focusing on the enzymatic process. Followed by, presented the different kinds of the used enzymes that differ according to the pectin forms. After that, the chapter displayed information about the various strains of microbes that can produce pectinolytic enzymes. Finally showed the importance of these enzymes and their industrial applications.

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Section 3

Pectin Applications

Chapter 6

Pectin-Based Scaffolds for Tissue Engineering Applications

Anna Lapomarda, Aurora De Acutis, Carmelo De Maria and Giovanni Vozzi

Abstract

Tissue engineering (TE) is an interdisciplinary field that was introduced from the necessity of finding alternative approaches to transplantation for the treatment of damaged and diseased organs or tissues. Unlike the conventional procedures, TE aims at inducing the regeneration of injured tissues through the implantation of customized and functional engineered tissues, built on the so-called 'scaffolds'. These provide structural support to cells and regulate the process of new tissue formation. The properties of the scaffold are essentials, and they can be controlled by varying the biomaterial formulation and the fabrication technology used to its production. Pectin is emerging as an alternative biomaterial to non-degradable and high-cost petroleum-based biopolymers commonly used in this field. It shows several promising properties including biocompatibility, biodegradability, non-toxicity and gelling capability. Pectin-based formulations can be processed through different fabrication approaches into bidimensional and three-dimensional scaffolds. This chapter aims at highlighting the potentiality in using pectin as biomaterial in the field of tissue engineering. The most representative applications of pectin in preparing scaffolds for wound healing and tissue regeneration are discussed.

Keywords: pectin, tissue engineering, scaffolds, bioprinting, biofabrication, tissue regeneration

1. Introduction

Tissue engineering (TE) is an interdisciplinary field whose first definition dates back to 1987. It combines the knowledge from different research areas including medicine, material science and engineering to develop engineered biological substitutes able to restore, maintain or improve tissue functions [1]. TE was introduced from the necessity of finding alternative methodologies to organ transplantations due to their increasing demand in clinical medicine. Furthermore, TE emerged as a promising approach to overcome the limitations of the conventional surgical approaches for the treatment of tissue damages caused by injuries, diseases and congenital disorders [2, 3]. These surgical procedures are based on replacing the injured tissues or organs with a healthy one harvested from the same patient (autograft), or a compatible donor (allograft). Although these approaches have been revolutionary and lifesaving, there are still some drawbacks that need to be addressed. The surgical procedures used to harvest both autografts and allografts are often invasive and painful. The risk of post-surgical limitations in

the donor's body due, for example, to infections and hematomas is, in fact, quite high. Moreover, when allografts are transplanted, the chance of inflammatory and immune responses in the patient body together with the transmission of diseases from the donor to the patient is significant [4].

TE aims at overcoming the complications associated with the conventional techniques used during organ transplantation by inducing the complete regeneration of the damaged tissues instead of replacing them [2, 3]. Several approaches to promote *de novo* tissue formation have been implemented in TE so far. These are mainly based on the use of biodegradable and biocompatible engineered tissues, based on the so-called '*scaffolds*'. A scaffold is a structure that provides temporary mechanical support and a guiding template to cells during the synthesis of new tissue. With the desired shape, architecture and functions. Concurrently, the scaffold biodegrades leaving space for new tissue in-growth. Notably, the biodegradability of the scaffold is what differentiates it from permanent implants. The complete biodegradation of the scaffolds prevents, the need for additional surgical interventions to remove it or, eventually, substitute it. The scaffold can be directly implanted into the injured site to induce the regeneration of the tissues *in vivo.* Otherwise, prior to implantation, the scaffold can be initially cellularized with cells isolated from the patient, subsequently cultured *in vitro* to synthesize tissues that will finally be transplanted into the defect to restore its functions (**Figure 1**). In this case, scaffolds can be further

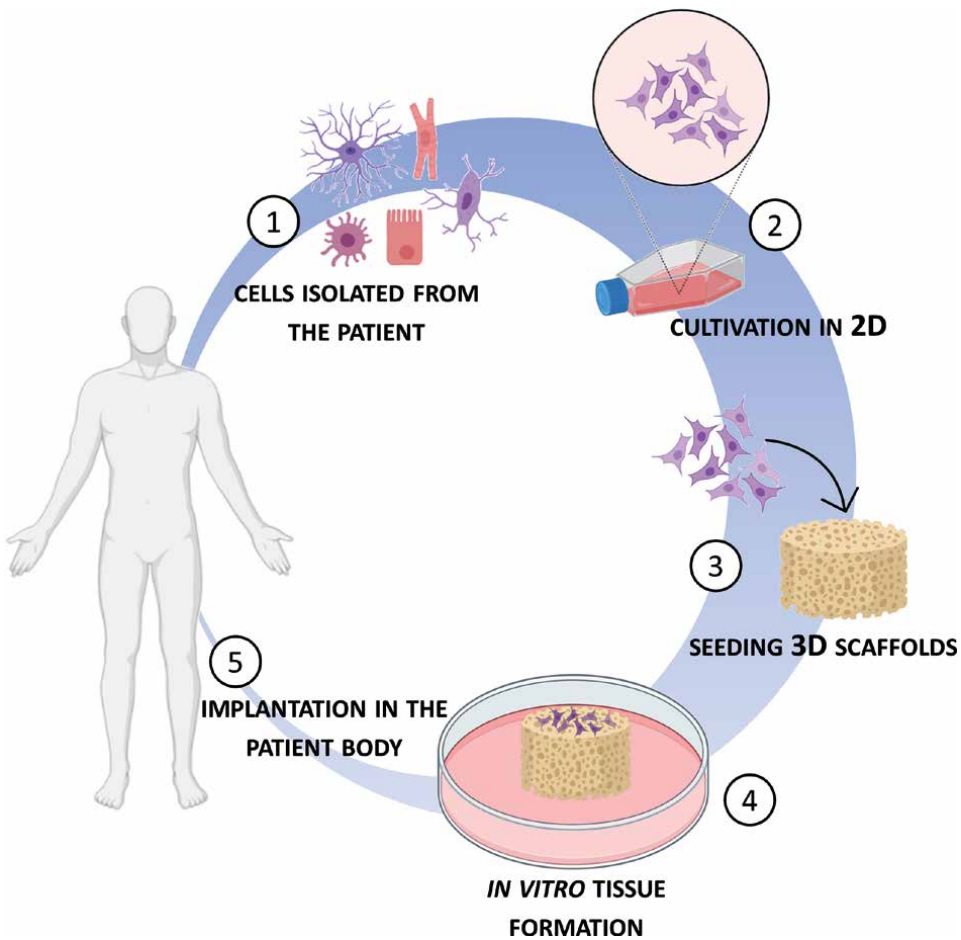

Figure 1.
Illustration of TE paradigm (figure created with BioRender.com).

cultivated in bioreactors, namely, devices able to apply biophysical stimuli to cells (e.g., mechanical or chemical) to better mimic the dynamic physiological conditions. In both approaches, the scaffold can be loaded with drugs, growth factors, micro- and/or nano-particles to further facilitate the recovering capabilities of tissues [5, 6].

The scaffold plays an essential role in regulating the process of new tissue formation. An ideal scaffold should be biocompatible and should degrade with kinetics compatible with the rate of tissue regeneration. It should be highly porous (< 75% [7]) with adequate pore size to promote cell migration/scaffold colonization and nutrient transfer throughout the scaffold. A scaffold should mimic the features of biological tissues in terms of topological properties (e.g., shape, size), mechanical properties (e.g., stiffness), and the biochemical processes that control and regulate the functionalities of the tissues. Moreover, it should not alter the normal functions of cells, which should adhere, migrate and proliferate within the scaffold before producing new tissue [5, 6, 8, 9]. Depending on their applications, scaffolds with different shapes, compositions and properties have been developed so far.

The biomaterial formulations used to produce the scaffold strongly affect its properties [10, 11]. Thus, the selection of the proper biomaterial formulation is pivotal for inducing the regeneration of the tissue in a controlled manner avoiding any undesired side-effects (e.g., cytotoxicity, apoptosis, carcinogenicity). The most used biomaterial formulations in TE are mainly based on synthetic biopolymers, natural biopolymers and composites [12, 13]. Synthetic biopolymers, like polycaprolactone, can be produced on a large scale under controlled conditions with predictable and reproducible physicochemical properties (e.g., mechanical properties, biodegradability) [6, 14, 15]. However, many synthetic biopolymers that have been developed so far are mainly derived from petroleum and coal, which make them not compatible with the environment [16]. Natural biopolymers include animal-derived proteins (e.g., gelatin, hyaluronic acid, collagen, silk) and animal- and vegetal-derived polysaccharides (e.g., cellulose alginate, chitosan). One of the advantages of this class of biopolymers is their biological similarity to native tissues which is beneficial for supporting cell functionalities (e.g., cell adhesion). Nonetheless, the use of animal-derived biopolymers may be associated with a high risk of transmission of diseases from animal to patient [10, 17, 18]. Therefore, the use of naturally occurring biopolymers from vegetal sources represents an attractive alternative to overcome these limitations. Moreover, they represent an ecological alternative to synthetic biopolymers in the preparation of sustainable and green scaffolds.

In recent years particular attention has been paid to the adoption of methodologies to derive biopolymers from renewable sources, such as industrial by-products, such as pectin from fruit pomace produced from the fruit processing industry [19] and cellulose nanofibers obtained from paper waste [20]. The application of more ecologically viable biomaterials in TE may, in fact, strongly contribute to reduce the polluting impact of producing and using un-recyclable synthetic biopolymers. Among the renewable and natural biopolymers, pectin is gaining particular attention in TE for its advantageous properties including biocompatibility, biodegradability and non-toxicity [21, 22]. In addition, the versatility in processing pectin-based formulations allows to produce scaffolds with diverse properties and for different applications (Section 2).

This chapter aims at highlighting the applications of pectin as the building block of bidimensional (2D) and three-dimensional (3D) scaffolds for TE applications. With this aim, in Section 2 the properties of pectin as biomaterial are provided. Section 3 reports the most representative applications of pectin-based formulations for producing scaffolds for tissue regeneration in the shape of 2D films for wound healing and 3D scaffolds for tissue regeneration.

2. Properties of pectin as a biomaterial for TE applications

Pectin shows several remarkable properties as a biomaterial. It is biocompatible and biodegradable, and it is soluble in cytocompatible and non-toxic solvents (such as water). Pectin is a versatile biomaterial as its physical properties can be facilely tuned due to the presence of several functional groups (e.g., carboxylic groups) that can serve as binding sites for other functional groups, biomolecules and drugs [21–23]. It is a low-cost biomaterial due to its ubiquity in nature, and this can strongly reduce the costs associated with the development of engineered tissues.

Pectin can form hydrogel due to the ability of its macromolecules to absorb and retain large volumes of water. This unique property makes pectin a suitable candidate to produce a natural extracellular matrix, which naturally surrounds cells. Furthermore, due to the possibility to be processed under sterile and physiological conditions (i.e., the aqueous environment at 37°C), pectin enables to encapsulate cells within its matrix to produce cell-laden scaffolds [23, 24].

Pectin tends to dissolve under physiological conditions, therefore physicochemical approaches are required to stabilize pectin-based scaffolds. These are mainly based on the use of physicochemical crosslinking approaches which consist of the formation of a stable network of links among the pectin molecules. This network reduces the interactions of pectin molecules with water and prevents the disruption of pectin-based scaffolds. For example, the most employed approach to form water-insoluble scaffolds of low-methoxyl pectin is based on the use of divalent cations (e.g., Ca^{2+}) that interact with the carboxylic groups of pectin forming the so-called 'egg box' structure [21]. Notably, the crosslinking treatments should also be cytocompatible (under specific conditions/concentrations), and should not interfere with the capability of pectin to encapsulate cells [25].

One of the major drawbacks that limit the application of pectin as a biomaterial for TE applications is its low cell adhesivity due to the lack of sites for cell adhesion (such as arg-gly-asp (RGD) sequences). Therefore, pectin is often combined/blended with other biopolymers or biomolecules to enhance its bioactivity [21, 26].

3. Applications of pectin in TE

Pectin-based formulations have been processed through different fabrication approaches into scaffolds with various shapes for different applications. In particular, pectin has been mainly used for the production of 2D films for wound healing, and 3D scaffolds for tissue regeneration. **Figure 2** provides a graphical overview of the main applications of pectin in TE.

3.1 2D patches for tissue regeneration

One of the applications of pectin-based formulations is the preparation of 2D hydrogel patches for the treatment of wounds. These patches provide mechanical support to cells during the process of new tissue formation, and an antibacterial barrier preventing eventual infections. Moreover, the hydrophilic pectin molecules in the film can react with the fluids of the wound forming a soft gel. The presence of a gel allows to maintain a moist environment in the wound. This helps to remove or control secretions from the wounded tissue and in turn facilitates the healing process. The regeneration of the damaged tissue can be further promoted by the incorporation of bioactive molecules such as drugs (e.g., antibiotics) and/or growth factors within the pectin patches [21]. The controlled and prolonged release

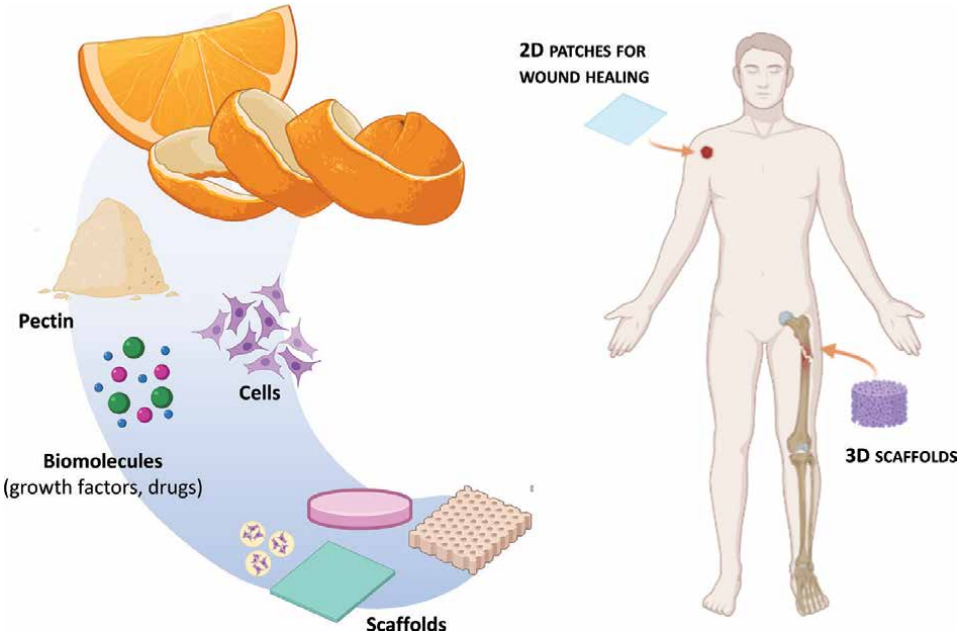

Figure 2.
Illustration of the application of pectin (derived from citrus fruits) for the production of scaffolds for TE applications (created with BioRender.com).

of these molecules directly in the damaged site can actively contribute to decreasing the risk of infections and accelerating the formation of new tissue. As mentioned in Section 2, pectin is often combined with other biopolymers to enhance its bioactivity and also to modulate the physical properties (e.g., tensile strength) of the final patch.

Pectin-based patches for wound healing reported in the literature so far are principally obtained in the shape of non-porous films and porous membranes, as detailed described in the following Sections 3.1.1 and 3.1.2, respectively.

3.1.1 Pectin-based films

Pectin-based films are generally 2D, non-porous and flexible substrates able to retain large volumes of water within their matrix. One of the approaches used to produce these films is the so-called 'solvent casting'. In this approach, a pectin-based solution is initially poured into a mold, and the solvent is subsequently let to evaporate leaving a 2D non-porous film (**Figure 3**).

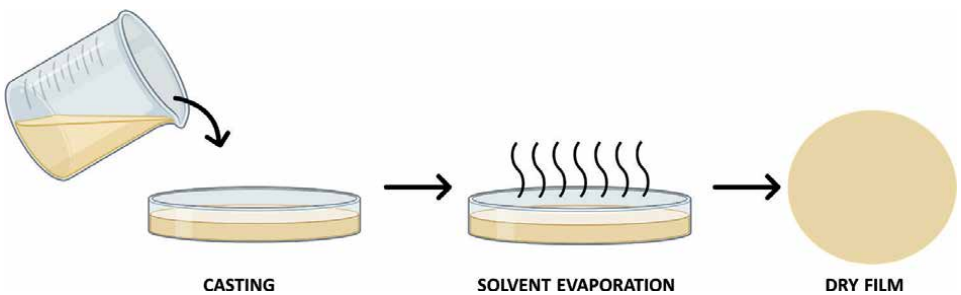

Figure 3.
Illustration of the solvent casting approach (created with BioRender.com).

Pectin-based patches produced with this approach support cell adhesion and proliferation and accelerate the processes occurring during the formation of new tissue [27–30]. Moreover, films with high toughness and stretchability can be produced with solvent casting, and these can be potentially used as pectin-based patches for load-bearing tissues (e.g., cartilage, tendon) [28]. In addition, pectin-based patches for a controlled drug into the targeted tissue were also produced by incorporating drugs in the pectin matrix [30, 31].

3.1.2 Pectin nanoporous membranes

Nanoporous membranes based on pectin have been mainly obtained through electrospinning. This approach allows to produce highly porous and flexible patches starting from pectin-based/polymer solutions subjected to an external electric field. A standard electrospinning apparatus is illustrated in **Figure 4**. It generally consists of (i) a syringe pump containing the polymer solution, (ii) a metallic needle through which the polymer solution is ejected, (iii) a high voltage power supply (in the range of tens of kVolts), and (iv) a grounded collector (usually a metal plate). When a drop of the polymer solution is extruded through the needle, the high electric forces in the space between the needle and the collector induce its stretching and the formation of fibers from a few nanometers to microns in diameters [32]. These fibers are therefore deposited and collected on the collector forming a non-woven fibrous membrane after complete evaporation of the solvent (**Figure 4**).

Pectin-based patches obtained by this approach show several advantageous properties for TE applications. The random organization of electrospun pectin fibers together with the hydrogel nature of pectin enables to mimic the nanoscale organization of the native extracellular matrix. Furthermore, the high porosity and high surface-to-volume ratio typical of electrospun patches promote cell migration and nutrient diffusion within the scaffold, which is beneficial for the process of new tissue formation [33]. Nevertheless, it is quite challenging to produce electrospun structures from pristine pectin due to some intrinsic molecular properties of pectin (such as insufficient chain entanglement) that disable the fiber formation [34]. Thus, to improve its electrospinning ability, pectin is often chemically modified [35, 36] and/or combined with other biodegradable biopolymers such as poly(ethylene oxide) [34], polyhydroxybutyrate [37] that work as carrier polymer to induce the formation of stable fibers.

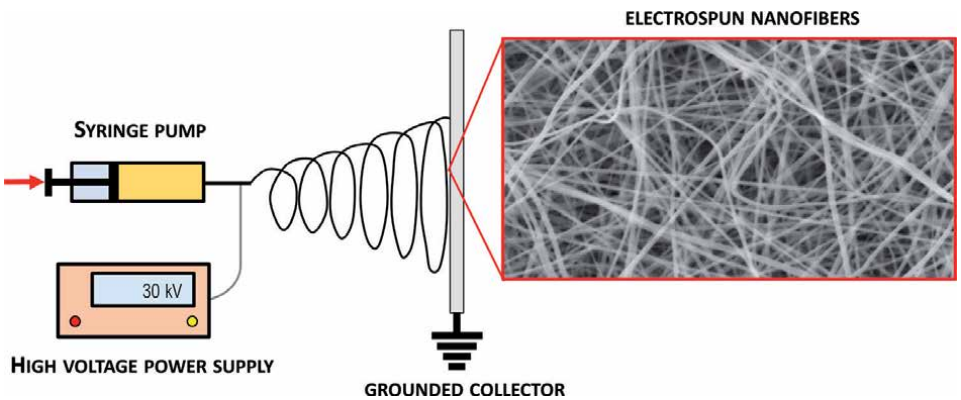

Figure 4.
Illustration of an electrospinning setup with a magnification of the electrospun nanofibers on the collector (image obtained with scanning electron microscopy).

Pectin-based nano-fibers find application for the preparation of films/structures that can be potentially used as patches for wound healing of soft tissues [35–37] (e.g., vascular tissue [35], retinal tissue [37]). In addition, drugs (such as antibiotics [38, 39]) and particles (such as argentum ions for antibacterial purposes [38]) can be successfully loaded in these structures obtaining patches for a local and controlled release of drugs directly into the wound.

3.2 3D pectin scaffolds

Pectin-based formulations can be further processed to obtain 3D scaffolds able to mimic the complex architecture of biological tissues. 3D pectin-based scaffolds have been principally obtained in the shape of porous 3D sponges and 3D bio-printed scaffolds.

3.2.1 Pectin-based sponges

Sponges are comparable to foams with an interconnected network of pores. This type of architecture is beneficial for cell penetration and scaffold colonization, while ensuring adequate diffusion of nutrients to cells within the scaffold. Moreover, a highly porous scaffold with open and connected pores is of critical importance as it allows for the diffusion of nutrients and waste products through the scaffold [6, 7].

Pectin-based sponges are mainly obtained by freeze-drying, also known as lyophilization. This technique consists in freezing a polymer solution followed by the evaporation of the frozen solvent by sublimation. Thus, a solid polymer matrix with numerous and interconnected pores is obtained (**Figure 5**). Before freezing, polymer solutions are generally poured into molds to produce porous scaffolds with the desired shape.

Pectin-based sponges have been principally used to produce scaffolds for wound healing and tissue regeneration. For example, sponges obtained with pectin-based formulations have been used as scaffolds for different types of tissues including cartilage [40, 41], skin [42], and bone [43]. The high hydrophilicity of pectin molecules and the interconnected porosity enables these sponges to entrap a large volume of water creating a 3D hydrogel-based environment that can mimic the natural extracellular matrix [40, 41]. Furthermore, this provides and stabilizes a moist environment for wounds that strongly contributes to accelerating the healing of the wounds [44].

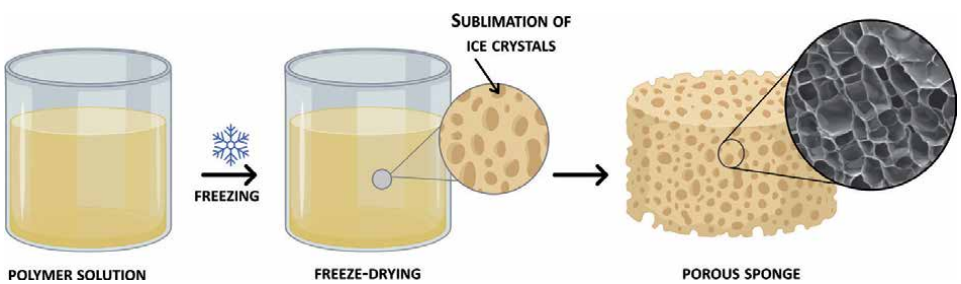

Figure 5.
Schematic of the process for obtaining cylindrical porous sponges was obtained by freeze-drying. Magnification of the porous sponges obtained by scanning electron microscopy (image created with BioRender.com).

3.2.2 Complex shaped pectin-based scaffolds

Producing scaffolds with a customized architecture and by automated and high reproducible approaches is one of the main challenges of TE. The development of pectin-based scaffolds with patient-specific architecture may boost their clinical applications.

Pectin-based scaffolds with complex shapes have been principally obtained by extrusion-based bioprinting so far. Extrusion-based bioprinting is one of the most widely used technology in TE due to its simplicity and versatility in processing a large variety of biomaterials, cells and biomolecules. An extrusion-based bioprinter usually consists of a movable cartridge containing the biomaterial formulation (called '*ink*') and of a movable deposition stage (**Figure 6**). Before bioprinting, the architecture of the scaffolds can be designed by a computer-aided design (CAD) software, or it can be derived from patient medical images acquired, for example, by computed tomography scans or magnetic resonance imaging. The 3D model of the scaffold is subsequently sliced by a computer-aided manufacturing (CAM) software in bioprinting paths and finally converted to a printable code file (called '*G-code*') [45, 46]. During the bioprinting process, the ink is extruded onto the deposition stage following the preprogrammed paths contained in the G-code, in a layer-by-layer process.

The application of pectin-based inks in extrusion-based bioprinting is relatively recent compared to the other fabrication approaches described in the previous sections. Pectin solutions are often not suitable to be processed through extrusion-based bioprinting and structures with poor shape fidelity are often obtained. The first application of pectin as ink for extrusion-based bioprinting dates back to 2017. In this case, pectin was combined with another biopolymer (Pluronic F-127), and complex-shaped scaffolds were bioprinted [47, 48]. Cells were successfully loaded within this formulation and 3D bioprinted to produce living 3D constructs [24]. From that moment, other pectin-based inks have been developed and optimized to produce 3D scaffolds with high shape fidelity [49–51]. For example, pectin-based scaffolds with more complex shapes such as a human ear and nose shape for cartilage tissue regeneration were successfully obtained (**Figure 6**) [41].

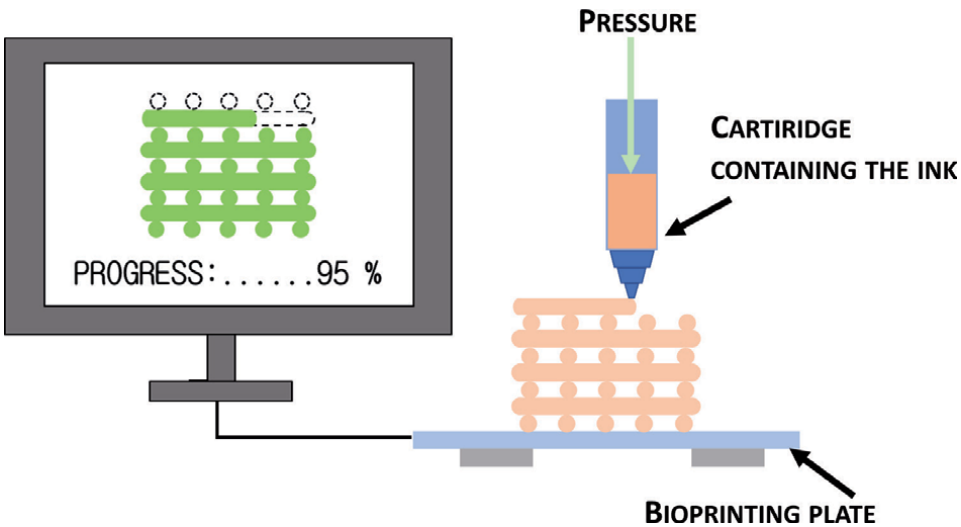

Figure 6.
Schematic of extrusion-based bioprinting.

4. Conclusions

TE represents an alternative approach to conventional surgical techniques used to treat damaged, injured or diseased tissues or organs. This approach is based on the use of tissue-mimicking and biodegradable constructs, based on the so-called 'scaffolds', able to restore, maintain or improve tissue functions. The physicochemical properties of the final scaffold play a key role in the process of new tissue formation. The selection of the proper biomaterial formulation is therefore essential. Recently, renewable biomaterials derived from industrial by-products are finding increasing application in TE as an alternative to petroleum-derived and unrecyclable polymers. In this regard, pectin, a polysaccharide commercially derived from citrus peel and apple pomace (both by-products of the food processing industry), is gaining attention in TE due to its biocompatibility, biodegradability and non-cytotoxicity. Diverse pectin-based formulations have been developed and employed for the fabrication of functional scaffolds for TE applications.

This chapter presented the most representative applications of pectin-based formulations for the fabrication of scaffolds for TE applications. In particular, by properly processing these formulations through specific fabrication techniques is possible to produce pectin-based scaffolds with different features: from 2D non-porous films (obtained by solvent casting) to 3D scaffolds with patient-specific shape (obtained by extrusion-based bioprinting). Although pectin shows diverse advantageous properties as biomaterial, its application in clinical practice is still under investigation. The increasing number of studies on the preparation of biocompatible pectin-based formulations may strongly boost the employment of this polysaccharide in the fabrication of sustainable scaffolds for future TE applications.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 7

Pharmaceutical Applications of Pectin

Olufunke D. Akin-Ajani and Adenike Okunlola

Abstract

Pectin, a natural ionic polysaccharide found in the cell wall of terrestrial plants undergoes chain–chain association to form hydrogels upon addition of divalent cations. Based on its degree of esterification, pectin has been classified into two main types. The high methoxyl pectin with a degree of esterification greater than 50%, which is mainly used for its thickening and gelling properties and the low methoxyl pectin, which is widely used for its low sugar-content in jams, both applications being in the food industry. Pectin is mostly derived from citrus fruit peels, but can also be found in other plants such as waterleaf leaves, cocoa husk, and potato pulps. Pectin has been used as an excipient in pharmaceutical formulations for various functions. This chapter will focus on the various applications to which pectin has been used in the pharmaceutical industry.

Keywords: Pectin, Degree of Esterification, Drug Delivery, Polymer Matrix, Excipients

1. Introduction

In the pharmaceutical industry, plants and plant products are continually used as sources of drugs and excipients. In particular, plant-derived polymers have contributed significant roles in drug delivery systems where they function as excipients [1]. Excipients refer to the non-pharmacological ingredients that are required to convert the Active Pharmaceutical Ingredient (API) into a dosage form. The International Pharmaceutical Excipients Council (IPEC, 1995) defines excipients as all substances contained in a dosage form other than the active substance or finished dosage form, which have been appropriately evaluated for safety and are included in a drug delivery system [2]. Excipients are included in drug delivery systems to assist in processing during manufacture, protect, support, enhance stability, bioavailability and patient acceptability, help in product identification, or enhance any other aspects of the drug delivery system's overall safety and effectiveness during use or storage [2–4]. Far from being just a random combination of ingredients, a pharmaceutical formulation is a well-rationalized formulation designed to satisfy quality and performance. Excipients are essential in the drug development process, as well as the formulation and administration of stable dosage forms [2]. Excipients are required in drug formulations to guarantee the potency, safety, predictability and reproducibility of the release of the API as well as its palatability and suitability for the patients [3].

The interest in excipients of plant origin over semi-synthetic or synthetic excipients is not far-fetched: low toxicity, relative abundance, cost-effectiveness

and non-irritant nature make them preferable to others sources [5]. Plant-based polymeric excipients can be used in different pharmaceutical formulations where they act as diluents or bulking agents, thickeners, binders, disintegrants, suspending agents, emulsifiers, film formers, matrix formers, release modifiers, sweeteners and mucoadhesive polymers [6–9]. These natural polymers would have to fulfill the requirements of an ideal excipient to be successful candidates for use as excipients in various formulations for pharmaceutical use. The requirements for an ideal excipient includes being pharmacologically inert, non-toxic and non-irritant as well as being non-reactive with drug or with other substances present in the formulation and the packaging. In addition, they must be easy to handle, cost-effective and readily available for the sustainable manufacture of the pharmaceutical product. Numerous plant polymers fulfill many of these requirements and have found application in pharmaceutical formulations. These include Inulin; a polysaccharide obtained from plant sources like; onion, garlic, artichoke and chicory, starches which are polymeric carbohydrates with large glucose units joined by glycosidic bonds, gums, and mucilage such as: acacia gum, tragacanth gum, locust bean gum, okra mucilage, seaweed polysaccharides which include carrageenan, agar and alginates, microbial polysaccharides such as: xanthan gum and pullulan obtained by the fermentation of carbohydrate products by specific bacteria or fungus, and polysaccharides of the plant cell wall with cellulose, hemicelluloses, pectin being the main polymers of this group [10–16].

Pectin, a structural heteropolysaccharide, is considered the second most abundant component of the cell wall of all terrestrial plants [17, 18]. It is a hydrophilic polymer that is biodegradable, biocompatible and non-toxic, making it a good biomaterial for packaging, coating and various pharmaceutical applications. Pectin is normally produced during the initial stages of growth of the primary cell wall and constitutes about one-third of the dry substance of the cell wall of some monocotyledonous and dicotyledonous plants [19]. A white to light brown powder, pectin is found in numerous fruits and vegetables. The main raw materials for pectin production are dried citrus peels or apple pomace, both by-products of juice production that are often discarded as waste. Alternative sources of pectin extraction include sugar beet waste from sugar manufacturing, mango waste from mango canning factories and sunflower seeds used for extracting edible oil, waterleaf leaves, cocoa husk, and potato pulps [20–23].

Pectin is the methylated ester of polygalacturonic acid which contains 1, 4-linked α-D-galacturonic acid residues and a variety of neutral sugars like arabinose, galactose, rhamnose and lesser amounts of other sugars [24, 25]. It can be classified into different types based on the degree of esterification or the number of methoxy groups that substitutes the carboxylic acid moiety on the galacturonic acid residues [26]. The degree of esterification influences the gelation mechanism, processing conditions and properties of the pectin [18, 27]. High methoxyl pectin is primarily used for gelation and has a degree of esterification greater than 50%. It requires a large amount of sugar and is acid-sensitive. Because of hydrogen bonding and hydrophobic interactions between the pectin chains, high methoxyl pectin forms a gel at low pH and a high concentration of soluble particles [28]. Low methoxyl pectin has a degree of esterification of less than 50% and is widely used in the food industry to form low sugar jams since it does not require a large amount of sugar for gelation. It shows less sensitivity toward acidity and requires Ca^{2+} ions to form gel [29]. Low methoxyl pectin is generally formed by the de-esterification of high methoxy pectin using acids, alkali, pectin methylesterase and ammonia in alcohol or concentrated aqueous ammonia. Monovalent cation i.e. alkali metal salts of pectin is normally soluble in water while di- and trivalent cations are partially or completely insoluble in water. When dissolved, pectin decomposes rapidly by

de-esterification or depolymerization. The rate of decomposition depends on the pH and temperature of the solution. The maximum stability of pectin is at pH 4 [30]. Low pH and high temperature increase the rate of degradation due to hydrolysis of the glycosidic linkage. At alkaline pH, pectin is rapidly de-esterified and degraded even at room temperature [31].

In this Chapter, the sections that follow would review in detail, some important pharmaceutical applications of pectin and possible modifications to enhance the future uses of pectin in pharmaceutical formulations.

2. Pharmaceutical uses of pectin

2.1 Drug delivery systems

The polymer pectin has been put to several uses since its discovery over 200 years ago. Though its major application has been in the food industry where it has been used as a gelling agent, emulsifier, stabilizer, thickener, and more recently as a food packaging material, where they as used as edible films on fruits and vegetables etc. The most important use of pectin is based on its ability to form gels, hence its potential as an excipient; pectin has been used as a binding agent [32, 33] in tablets, carrier for drug delivery to the gastrointestinal tract from matrix tablets, and as a controlled-release matrix in tablet formulations [34–36]. It has also been used as a sustained release drug delivery system in gel beads prepared by the ionotropic gelation method [19, 37, 38], colon-specific drug delivery vehicle [39], and film-coated dosage forms. Gel formation is caused by hydrogen bonding between free carboxyl groups on the pectin molecules and between the hydroxyl groups of neighboring molecules [40]. Most of the unesterified carboxyl groups in pectin occur as partially ionized salts in a neutral or very slightly acid dispersion of pectin molecules. [41]. Those that are ionized produce a negative charge on the molecule, which together with the hydroxyl groups causes it to attract layers of water [38]. Because of their negative charge, the repulsive forces between these groups can be strong enough to preclude the creation of a pectin network. When acid is added, the carboxyl ions are converted to mostly unionized carboxylic acid groups [38]. The attraction between pectin and water molecules is lowered by a reduction in the number of negative charges, which also lowers the forces of repulsion between pectin molecules. Sugar further decreases the hydration of the pectin by competing for water [41]. These conditions decrease the ability of pectin to stay dispersed. When cooled, the unstable dispersion of less hydrated pectin forms a gel, a continuous network of pectin holding the aqueous solution. High methoxyl pectin produces gels with sugar and acid. Unlike Low methoxyl pectin, high methoxyl pectin does not contain sufficient acid groups to gel or precipitate with calcium ions, although other ions such as aluminum or copper cause precipitation under certain conditions [25]. The degree of esterification (DE) affects the rate at which gel formation takes place [38]. A higher DE causes a more rapid setting. Slow-set pectins (with DE 58–65%) gel at lower soluble solids and greater levels than rapid-set pectins (DE > 72 per cent). Low methoxyl pectins require the presence of divalent cations (usually calcium) for proper gel formation [38].

2.2 Bioadhesive systems

The ability of pectin to absorb water, swell and form bioadhesive bonds with biological tissue has found application in the preparation of mucoadhesive formulations such as patches [42]. Pectin has also been found useful as a demulcent in

Brand Name	Ingredients/strength	Dosage form	Manufacturer
Berry Breezer Throat Drop	7 mg/1	Lozenge	Topco Associates USA
Burts Bees Throat Soothing	10.5 mg/1	Lozenge	L. Perrigo Company USA
CVS Clean Label Throat Relief Pops	12 mg/1	Lozenge	CVS Pharmacy USA
Grape Throat Relief Lollipop	10 mg/1	Lozenge	Topco Associates USA
Little Remedies Sore Throat Pops	5.4 mg/1	Lozenge	Medtech Products Inc. USA
Ludens Assorted Flavors	2.8 mg/1	Lozenge	Prestige Brands Holdings, Inc. USA
Sundown Honey Soothers Lollipops	0.1 g/100 g	Lozenge	The Nature's Bounty Company USA
Throat Coat Lemon Ginger Echinacea	5 mg/4.2 g	Lozenge	Traditional Medicinals, Inc. USA
Axcel Kaopec Suspension	Pectin (20 mg/5ml) + Kaolin (1 g/5ml)	Suspension	Kotra Pharma (M) Sdn. Bhd.
Benylin DM With Pectin Freezer Pops	Pectin (150 mg/unit) + Dextromethorphan hydrobromide (7.5 mg/unit)	Liquid	Mcneil Consumer Healthcare Division of Johnson & Johnson Inc
Cepacol Sore Throat and Coating	Pectin (5 mg/1) + Benzocaine (15 mg/1)	Lozenge	Reckitt Benckiser
Cepacol Sore Throat Plus Coating	Pectin (5 mg/1) + Benzocaine (15 mg/1)	Lozenge	Combe Incorporated
Diaret Liq	Pectin (150 mg/30 mL) + Kaolin (3.078 g/30 mL)	Liquid	Produits Francais Labs Inc. Canada
Diaret Tab	Pectin (45 mg/tab) + Aluminum hydroxide (70 mg/tab) + Attapulgite (350 mg/tab) + Zinc phenolsulfonate (30 mg/tab)	Tablet	Produits Francais Labs Inc. Canada
Diban Cap	Pectin (71.4 mg) + Atropine sulfate anyhdrous (9.7 mcg) + Attapulgite (300 mg) + Hyoscyamine sulfate (0.0519 mg) + Opium (12 mg) + Scopolamine (3.3 mcg)	Capsule	Wyeth Ayerst Canada Inc.
Orabase Paste	Pectin (13.3%) + Carboxymethylcellulose sodium (13.3%) + Gelatin (13.3%)	Paste	Convatec Inc.
Organix Complete	Pectin (1.7 mg/1) + Levomenthol (2.5 mg/1)	Lozenge	Pro Phase Labs, Inc.
Herbon Berry Buddies	Pectin (10 mg/1) + *Echinacea purpurea* (50 mg/1)	Lozenge	Purity Life Division of SunOpta

Table 1.
Some commercial drug products containing pectin.

throat lozenges where it gives temporary relief for minor discomfort and protects irritated areas in sore mouth and sore throat [43]. The antihemorrhagic effect of pectin has also been utilized in wound healing as medical adhesives [44].

2.3 Disperse systems

Pectin has been shown to have foam stabilizing and emulsification potential since the protein and hydrophobic acetyl groups of pectin can act as anchors on the oil particle surface, thus decreasing the surface tension [45]. Other areas of use have been as an emulsifier in oil: water emulsions [46, 47], and as a viscosity enhancer in lipid digests [48]. Pectin slows gastric transit thus helps control energy intake and hence its use by weight-watchers, since the large water-binding capacity of pectin reduces contact between intestinal enzymes and food, thus prolonging gastric emptying half-life, allowing a marked reduction in quantity and frequency of eating [49]. Furthermore, its interaction with polyphenolic compounds leads to systemic anti-inflammation [50].

2.4 Health benefits

In the pharmaceutical industry, pectin has been used both for its health benefits and as an excipient. Pectin as an active agent was formerly used in diarrhea mixtures, in conjunction with kaolin and sometimes bismuth compounds, and in wound dusting powders and ulcer dressings where pectin appears to have some specific activity in promoting healing [25]. It has been found to have certain health benefits such as reducing cancer development, lowering blood cholesterol and blood glucose level through the different domains of the pectin structure, and stimulating the immune response [51–55].

2.5 Other applications

Pectin's application has spread to water treatment where it is used as a biosorbent to remove heavy metals [47] and in urinary excretion of toxic minerals such as lead, cadmium, strontium, or arsenic [56–58]. In cosmetics, it is used as a plasticizer, texturizer and adhesive [59], and in biomedical applications, where it is used as a biomaterial ink to fabricate patient-specific scaffolds when cross-linked with 3-glycidyloxypropyl trimethoxysilane (GPTMS) [60]. Some examples of drug products that contain pectin are presented in **Table 1**.

3. Material properties of pectin

Pectin is an important biomaterial that has numerous pharmaceutical applications. Its application, however, largely depends on its material properties such as degree of esterification (DE), degree of blockiness (DB), ash value and solubility. This section will focus on these properties and how each affects the application of pectin pharmaceutically.

3.1 Degree of esterification

The DE of pectin is the ratio of esterified D-galacturonic acid (GalA) groups to total GalA groups [34, 61]. Depending on the species, tissue, and maturity of the plant, the DE can have a wide range. In general, the structure of pectin is mostly composed of homogalacturonan (HG), regions (partially 6-methylated and

2- and/or 3-acetylated poly-α(1–4)-D-galacturonic acid residues), alternating with rhamnogalacturonan I (RG-I), regions (branched α(1–2)-L-rhamnosyl-α(1–4)-D-galacturonosyl chains substituted with side chains of mainly α-L-arabinofuranose and α-D-galactopyranose) [18, 62]. The interconnection of HG "smooth" (responsible for the gelling capability) and RG-I "hairy" (play a gel-stabilizing role) regions, in relative proportions determine the flexibility and rheological properties of the polymer in solution [63, 64]. The gelling mechanism of pectin is dictated by its degree of esterification (total methoxyl content) [65]. Pectin based on the DE can be classified as high methoxyl (HM) pectin with DE > 50% or low methoxyl (LM) pectin DE < 50%, which are either the conventionally demethylated or the amidated molecule [66–68]. The two groups of pectin gel by different mechanisms. To form gels, high methoxyl pectin requires a minimum amount of soluble solids and a pH of around 3.0.

HM pectins are generally hot water-soluble, thermally reversible, and often contain dextrose (a dispersion agent) to prevent lumping. Conversely, LM pectins produce gels independent of sugar content, are less sensitive to pH compared to the HM pectins, and require the presence of a controlled amount of calcium or other divalent cations for gelation [41].

The specific application to which pectin will be put is a function of its gelling behavior, which is dependent on its DE, the monosaccharide content (HG), and the spatial disposition of the cross-linking blocks (RG) [69]. While HM pectins have been used in tablet formulations as a binder, controlled-release matrix and taste masker through complexation with bitter molecules, the LM pectins have been used as sustained-release matrices in microspheres produced by ionotropic gelation [19, 53, 69].

3.2 Degree of blockiness

Pectin, an anionic cell wall polysaccharide through its non-methyl esterified galacturonic acid units, interacts with divalent cations [40, 47]. At pH values above the pKa of pectin (2.8 to 4.1), non-methyl esterified GalA residues can be negatively charged, giving pectin the ability to interact with cations [34, 70]. Thus, the lower the DE of pectin, the higher the number of non-methyl esterified GalA residues present, the higher the cation-binding capacity. Due to LM pectin's higher number of negatively chargeable carboxyl groups (non-methyl esterified carboxyl groups) compared to HM pectin, it exhibits a higher charge density, further showing that the cation-binding capacity of pectin increases with decreasing DE [40, 70, 71]. Studies have shown that regardless of the method used, a stronger and higher bound interaction occurs between pectin with decreasing DE and cations (Fe^{2+}, Zn^{2+}, or Ca^{2+}) [47]. Furthermore, the DE and the intramolecular distribution of the non-methyl esterified carboxyl groups within the pectin determine pectin's anionic nature and associated functionality [72, 73]. Interestingly, less described in the literature is the influence of the distribution pattern of non-methyl esterified GalA units on the cation-binding capacity of pectin compared to DE [47].

Daas et al. first quantified the relative occurrence of blocks of non-methyl esterified GalA units within a pectin chain as the degree of blockiness, DB [47, 74]. Apart from the DB, the absolute number of non-methyl esterified GalA units present in blocks can be expressed as the absolute degree of blockiness (DB_{abs}). Both parameters (DB and DB_{abs}) were established by exhaustive enzymatic degradation of pectin using endo-polygalacturonase (endo-PG) of *Kluyveromyces fragilis*, which required at least four consecutive non-methyl esterified GalA units to hydrolyze the linkage between two non-methyl esterified GalA units [70]. The DB is the proportion of galacturonic acid units (mono-, di-, and tri-) released by the enzyme to the total amount of non-methyl esterified GalA units, while DB_{abs} is the number of

GalA oligomers released in the endo-PG digest to the total number of GalA units in the pectin polymer, without adjustment of the DE [47, 74, 75]. Thus, to characterize the presence of blocks of non-methyl esterified GalA units, these parameters (DB and DB_{abs}) are used [70]. For most of the cations (divalent cations), the binding between them and pectin is known to follow the egg-box model [47]. The egg-box model of ´binding was mainly described for pectin-Ca^{2+} binding but assumed to be applicable for interaction between pectin and other divalent cations [76]. However, Assifaoui et al. [77] reported that the egg-box model was more appropriate for Zn^{2+} binding than Ca^{2+} as they found that Zn^{2+} interacts with both carboxyl and hydroxyl groups, comparable to the egg-box model, whereas Ca^{2+} binds only via carboxyl groups [40]. This egg-box model yields stronger gels [78, 79].

Applications to which a high DB is required would thus mean high cation-binding capacity and hence the use of LM pectins and the converse is true.

3.3 Ash value

The ash content of pectin is a valuable tool in determining the purity as well as the gel-forming capability of the polymer. The ash content of pectin has been found to increase as the yield of pectin decreases [80]. High levels of ash in pectin may be caused by elevated concentrations of negatively charged carboxylic groups of pectin and the counterions in solution during pectin precipitation [41]. However, for gel formation, low ash content ($\leq 10\%$) is a more favorable criterion as this will aid in determining the applicability of the polymer [47, 81, 80]. Ash content along with the anhydrouronic acid value of pectin has also been used to determine its purity [82, 83].

3.4 Solubility

Pectins are soluble in pure water. The solubility appears to depend on the valency of the cation salt; monovalent cation salts of pectin and pectic acids are usually soluble in water, while the di- and trivalent cation salts are weakly soluble or insoluble in water. Dry powdered pectin hydrates very rapidly when added to water, but tends to form clumps. These clumps are semidry packets of pectin within a highly hydrated outer coating. Dry mixing the powder with water-soluble carrier material can prevent the formation of clumps or by the use of specially treated pectin that has improved dispersibility [20, 83]. Studies have shown that pectin extracted with distilled water showed a high yield and low ash content when compared to other solvents [79]. High ash content and the drying process of the extracted pectin, however, may reduce the solubility of pectin [47]. It has been shown too that a decrease in the esterified carboxylic group reduced the solubility of extracted pectin; this insolubility of the extracted pectin is probably due to the presence of electrolytes in de-methylated pectic acid [47]. Thus, pectins with lower DE are less hydrophilic [69].

Dilute pectin solutions are Newtonian in behavior but at a moderate concentration, they exhibit the non-Newtonian, pseudo plastic behavior characteristics. Solubility, viscosity, and gelation are generally related. Whatever factors increase gel strength will increase the gelling tendency, viscosity, decrease solubility, and vice versa [84].

3.5 Antioxidant activity

Another property of pectin that could affect its application is its antioxidant activity. However, there are limited studies to show how this property may be applied to either the food industry or the pharmaceutical sector [85].

4. Modifications of pectin for future applications

The presence of several hydroxyl and carboxyl groups distributed along its backbone as well as a certain amount of neutral sugars presented as side chains gives pectin the capability of producing a broad spectrum of derivatives with modified or new functional properties. Various methods used for pectin modification include substitution (alkylation, amidation, quaternization, thiolation, sulfation, oxidation, etc.), chain elongation (cross-linking and grafting) and depolymerization (chemical, physical, and enzymatic degradation). Saponification (a process catalyzed by mineral acids, bases, salts of weak acids and primary aliphatic amines) can also be used to modify pectin chemically. Modification induced by pH changes can produce new fragments that have their solubility and biological activities altered [86]. Enzymatic modification of pectin has been achieved by using endo-polygalacturonase (Endo-PG), resulting in highly selective and specific structural changes in the polymer backbone. This modification leads to the cleavage of glycosidic linkages between two non-esterified α-D-galacturonic acid residues inside the HG fragment, which is depolymerisation. The enzymatic modification method can alter the macromolecular structure of pectin and can yield modified pectin with newer and improved properties and functionalities [87].

A new hydrolyzed polyacrylamide-graft-sodium alginate (PAAmg-SA) and diclofenac sodium-loaded interpenetrating polymer network (IPN) beads of pectin were developed using the ionic gelation method. The results of the investigation verified that hydrolyzed PAAm-g-SA and pectin cross-linked with aluminum ion (Al^{3+}) and glutaraldehyde could form an optimal matrix material for the production of IPN beads to support the sustained release of diclofenac sodium [88]. In another study, for the nasal administration of tacrine hydrochloride (an anti-Alzheimer drug), mucoadhesive microparticles based on chitosan/pectin polyelectrolyte complexes were prepared. The microparticles were produced by spray drying followed by lyophilization and direct spray drying. The study thus demonstrated the potential of the chitosan/pectin polyelectrolyte complexes to function variously in mucoadhesive microparticles [89, 90]. The chitosan/pectin molar ratio influenced the water uptake and tacrine hydrochloride permeation [90, 91].

Emerging advanced manufacturing technology in the field of tissue engineering and pharmaceutical formulations is the use of 3D bioprinting technology. 3D printing is an additive manufacturing technology in which objects are constructed in a layer-by-layer manner achieved by heat fusion, ultraviolet light (UV), and chemical bonding [91]. Spritam®, a fast disintegrating orodispersible tablet containing levetiracetam for epilepsy was the first 3D printed drug product approved by the US Food and Drug Administration (FDA) in 2015 [91]. To sustain the manufacturing of these types of drugs using this new technique, biomaterials that are green and non-toxic, derived from renewable sources and can be processed through 3D bioprinting are being developed [42, 60]. Common techniques include powder bed printing, vat polymerization (VP), and fused deposition modeling (FDM) [92]. A major disadvantage of the FDM technology is the need to insert printing materials into a nozzle in the form of a solid filament, which is non-existing for many pharmaceutical materials, thus necessitating the transformation of pharmaceutical-grade materials, including active pharmaceutical ingredients (API), into FDM-suitable filaments using techniques like hot-melt extrusion (HME). However, thermolabile therapeutics are not suitable for extrusion via FDM, due to potential degradation concerns [93]. The use of bio-inks for extrusion-based bioprinting at room or body temperature has shown clinical potential in achieving personalized treatment [92]. For example, Long et al. developed a personalized 3D printed wound dressing composed of chitosan and pectin with the ability to

control dimensional properties such as thickness and pore size using an extrusion-based bioprinter [91, 92], while allowing for facile lidocaine incorporation for immediate pain relief [94]. Pectin from citrus peels has also been cross-linked with (3-glycidyloxypropyl)trimethoxysilane (GPTMS) through a one-pot procedure to obtain freeze-dried porous pectin sponges with varying porosity, water uptake, and compressive modulus [42]. The addition of GPTMS improved the printability of pectin due to an increase in viscosity and yield stress [95]. Without the use of any additional support material, three-dimensional woodpile and complex anatomical-shaped scaffolds interconnected with micro and macro pores were, therefore, bioprinted [96]. Thus showing the great potential of pectin cross-linked with GPTMS as biomaterial ink to fabricate patient-specific scaffolds that could be used to promote tissue regeneration *in vivo* [42]. In another study, gelatin, another natural biopolymer has had its rheological properties improved to aid its bio-printing performance by using pectin as a rheology modifier of gelatin and GPTMS as a gelatin-pectin crosslinking agent [95]. Pectin played a key role in increasing the viscosity and the yield stress of low viscous gelatin solutions as shown through investigation of the rheological properties, as well as bioprinting assessments [96]. Water stable, three-dimensional, and self-supporting gelatin-pectin-GPTMS scaffolds with interconnected micro- and macro- porosity were successfully obtained by combining extrusion-based bioprinting and freeze-drying which did not require any additional temperature control to further modulate the rheological properties of gelatin solutions [95]. Patient-centric dosage forms have been produced through additive manufacturing techniques, which enable its design with precise control over dimension and microstructure, factors that are known to ultimately play key roles in modulating drug release kinetics, a feat not achieved through compression; traditional manufacturing techniques [92, 96, 97].

5. Conclusions

Pectin over the years has "metamorphosed" from just being a gelling agent for the production of jam and confectionaries to a biomaterial with health benefits to being useful as an excipient in drug delivery systems and more recently even personalized 3D printed medicine. This is as a result of a better understanding of its structure, mechanism by which it gels, and its properties such as degree of esterification and degree of blockiness, which has aided its classification and application.

The pharmaceutical industry has a material that can be explored in different functional dimensions as the usefulness and functionality of pectin unfolds.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

Effect of Cross-Linking Agent on Mechanical and Permeation Properties of Criolla Orange Pectin

María Guadalupe García and Martin Alberto Masuelli

Abstract

Pectin from orange peel was extracted and cross-linked, applying different cross-linking agents to visualize any effect on its mechanical and permeation properties. Calcium chloride (II) and iron chloride (III) were the cross-linking agents. Besides, commercial pectin was also used to compare its properties with neat orange pectin. Tensile testing showed mechanical stiffness of the orange pectin matrix in the presence of cross-linking agents. Calcium ions better cross-linked the polymer matrix as shown by their highest tensile strength and elastic modulus, with moderate elongation at break. Iron ions showed a weaker cross-linking effect on the pectin matrix, improving the elastic modulus but retaining almost the same tension strength. Lower elongation at break concerning neat orange pectin was observed for cross-linked samples. Water uptake (WU) and water vapor permeation (WVP) of cross-linked samples had lower values than those of neat orange pectin. However, these results are still high compared with synthetic polymers. Finally, gas permeation assays were performed using N_2, O_2 and CO_2 gases, according to exchangeable gases in fresh fruits and vegetable packaging. Results showed a conveniently modified atmosphere effect by avoiding CO_2 permeation and stabilizing N_2 and O_2 selectivity.

Keywords: polysaccharides, pectins, crosslinking agent, mechanical, permeation properties

1. Introduction

Pectin is a structural heteropolysaccharide present in the primary cell walls of terrestrial plants. It can be obtained from renewable agriculture by-products and food processing industry wastes. These natural sources of pectin make it one of the most abundant biopolymers. It consists of D-galacturonic acid residues which possess carboxylic acid groups, some of which are methyl-esterified. The degree of esterification determines the solubility of pectin and its gelling and film-forming properties. Depending on the origin of pectin, the degree of esterification can vary from high methyl (HMP, up to 50 wt. % of carboxylic acid units are esterified) to low methyl (LMP, lower than 50 wt. % of carboxylic acid units are esterified) [1]. Pectin extracted from criolla orange (*Citrus sinensis*) was first studied and characterized by Masuelli et al. [2]. These authors reviewed the extraction methods and characterization techniques of pectin in solution or film configurations. The intrinsic viscosity of orange pectin from dilute solutions was evaluated to estimate the extracted polymer's molecular weight. Results obtained ranged from 56 to 93 kDa

for basic and acid hydrolysis processes, respectively. Thermal analysis (DSC and TGA) and FTIR spectroscopy were carried out for structure characterization of pectin films. Results showed the presence of T_g (glassy) and T_m (melting) temperatures for pectin, which vary accordingly with the extraction method in the range from 57 to 69°C and 101 to 128°C, respectively. Thermograms (TGA) depicted temperature resistance up to 150°C for all types of extracted pectins. From FTIR analysis, carboxylic acid groups and esterified methyl groups were observed as signals at 1750 cm^{-1} and 1650 cm^{-1}, respectively. Morphological analysis was also carried out by taking SEM images of the film surface. All pectin films showed a homogeneous dense surface without defects. Mechanical, water absorption, and water vapor permeation tests were also performed. All pectin films showed too little resistance to tension and elongation at break compared with commercial pectin. Water absorption was a moderate but fast process, reaching 25% of absorbed water at 60 min of initiating the test. Water vapor permeation was as high as 2.62–5.25 $g{\cdot}m^{-2}{\cdot}day^{-1}$. Other authors [3], explored the interplay of the degree of methyl esterification (DM), pH, temperature, and concentration on the macromolecular interactions of pectin in solution. They found two levels of organizing pectin structures in solution: (i) chain clusters with a radius of gyration ranging between 100 and 200 nm and (ii) single biopolymer chains with a radius of gyration between ≈6 and 42 nm. Besides, they found that chain flexibility increases with DM and acidic pH, whereas hydrogen bonding is the responsible thermodynamic driving force for cluster formation. High methyl pectin creates structures with less efficient packing.

On the contrary, low methyl pectin at pH 7 or higher can turn into more coiled chain conformations in the presence of counterions. Furthermore, the addition of salt to pectin solutions allows the formation of complexes between positively charged ions and negatively charged carboxylic acids, which is facilitated at pH 7 or higher because of deprotonation of carboxylic acid groups. These observations explain the cross-linking effect of positively charged ions on pectin structure and gel formation at higher pH and ion concentration [4].

On the other hand, it is well known that pectin films are obtained from aqueous solutions after slow solvent evaporation [1]. High molecular weight and low pH are required to facilitate the formation of coil entanglements responsible for film formation. The chain entanglements are supported by H-bonding interactions that give strength and physical resistance to the film. Kontogiorgos et al. [5] found that the strength of interactions and conformational changes on pectin during the transition from a liquid to a glassy state are the main factors influencing the physical properties of the solid-state system. However, in contact with aqueous environments, pectin films can absorb water, first swelling the polymer matrix and then dissolving it. Several authors have probed different methods of preparing water-resistant films. Cruces et al. [6] prepared multilayer films of pectin-beeswax/colophony-pectin varying the ratio between beeswax and colophony. This method reached water vapor permeation values (56×10^{-13} g m m^{-2} s^{-1} Pa^{-1}) almost ten times higher than the WVP value of polyethylene films (LDPE 5.8×10^{-13} g m m^{-2} s^{-1} Pa^{-1}). Gharsallaoui et al. [7] prepared composite films of pectin/sodium caseinate to improve the mechanical and water barrier properties of protein-free pectin. These authors found that pectin and protein are negatively charged at neutral pH (pH higher than the isoelectric point of a protein), which favors the formation of macroscopic segregated phases. However, even at high turbidity conditions, which demonstrated phase segregation, some positively charged residues on protein might interact with negatively charged groups on pectin, improving the mechanical and water barrier properties. Other authors have prepared insoluble films by cross-linking the pectin matrix using divalent or trivalent cations [4, 8]. Besides, there exist methods of cross-linking a polymer matrix by reacting it with bifunctional molecules such as glutaraldehyde to

perform covalent cross-linking [9]. Usually, cross-linking of the polymer matrix causes chain stiffness and, consequently, detriment of mechanical properties.

Nevertheless, cross-linking might improve solvent resistance, water vapor, and gas barriers. So, it is interesting to study the proper film formation conditions in the presence of a cross-linking agent to overcome the challenge of obtaining a robust film, easy to manipulate with improved mechanical and barrier properties. In this work, pectin from criolla orange (*C. sinensis*) was cross-linked with divalent calcium ions and trivalent iron ions to improve mechanical and permeation properties. Pectin solutions at pH3.2 were used to prepare films by the "casting" method. Calcium and iron salts were separately contacted with pectin films by submerging them into ions solutions at 40°C for 24 h. The influence of positively charged ions on film properties was analyzed by uniaxial traction, water uptake, water vapor barrier, and gas permeation experiments. Conclusions about structure-properties relationships were obtained.

2. Materials and methods

2.1 Cross-linked film preparation

Pectin (Pec) from criolla orange was dissolved in distilled water at ambient temperature under mechanical stirring at a concentration of 2 wt.% of solid with 1 vol.% of glycerin (GLY, Biopack Argentina). The pH of the pectin solution measured with a pH meter (Melter Toledo) was 3.2. After the pectin solution was homogeneous, it was spread on a leveled Petri dish and placed in an oven at 40°C for 24 h for slow solvent evaporation. Once a pectin film was formed, it was peeled off and submerged in a 0.1 wt.% $CaCl_2$ (Merck) solution at 40°C without stirring (stagnant conditions). Diffusion of calcium ions occurred by a driving force of concentration gradient, allowing a moderate cross-linking effect in the polymer matrix. A contact time of 24 h was probed to obtain cross-linked pectin films. After cross-linking time, the pectin film was washed with a hydroalcoholic solution several times to remove excess calcium salt on the film surface. Cross-linked pectin with calcium was named Pec-Ca, and it resulted in a transparent and handling film used for mechanical and barrier characterizations. The same procedure and salt concentration were used in the case of $FeCl_3$ salt (Merck), and the cross-linked film was called Pec-Fe. This last film was light brown and retained transparency and easy handling. Commercial pectin from citrus peel was supplied by Sigma Aldrich (galacturonic acid ≥74.0%, methoxy groups ≤6.7%), and it was used to prepare uncross-linked films. The same procedure as pectin from criolla orange was used to obtain the commercial pectin film, and it was called Com-Pec.

2.2 Mechanical characterization

CT3 Brookfield texture analyzer with a load cell of 50 kg and a resolution of 5 g was used to perform tensile strength assays at a speed of 5 $mm \cdot min^{-1}$ according to the ASTM D 882 requirements. For an experiment, samples were cut into rectangular pieces of 40 mm in length and 10 mm wide. Thickness was measured using a Köfer micrometer (precision ±1 μm). To ensure complete relaxation of the polymeric structure once the films were peeled off, they were placed in a humidity chamber for 24 h at a relative humidity of 40% and room temperature (25°C) before they were measured. Then, the experimental procedure was carried out under the same humidity and temperature conditions. Typical curves of tension (σ) versus elongation (ε) were built for each sample, and they were used to determine the values of the mechanical parameters, such as young's modulus (E; MPa), tension at break (σ; MPa), elongation

at break (ε; %), and Tenacity (T; kJ·m^{-3}). Young's modulus was calculated from the slope of the σ–ε curves when a linear relationship between them was observed. σ and ε were calculated as the final points on the curves; this behavior indicated that rupture of the sample occurred, and tenacity was calculated as the area under the σ–ε curves before rupture [10]. The reported results are the average values from at least three film samples. The σ–ε relationship is given by the following equation:

$$\sigma = E \times \varepsilon \tag{1}$$

where: $\sigma = F/A$ in MPa, F being the force in N and A the transversal area of the specimen in m^2.

$$\varepsilon = \frac{\Delta L}{L_0} \times 100 \tag{2}$$

where: ΔL is the change in length and L_0 is the initial length of the specimen, which was 20 mm.

2.3 Water uptake

Polysaccharides in general and pectin are hydrophilic polymers able to absorb water from the environment to the detriment of the films' physical integrity. It is a matter of science to find ways to prevent water absorption for expanding the field of biopolymers application. Even reducing water uptake under acceptable values would represent a contribution to broadening polysaccharide film applications, for example, for food packaging. In this study, water uptake (WU) was determined gravimetrically. Weights of completely dried samples were measured directly. Film specimens were introduced into bottles containing 20 mL of distilled water and shaken at ambient temperature (25°C). At intervals of 24 h, films were removed from the medium, dried to remove excess water, and immediately weighed. The water uptake of the cross-linked films was calculated according to the following Equation [11]:

$$WU = \frac{W_{24} - W_0}{W_0} \times 100 \tag{3}$$

where: WU is the value of water uptake (%), W_{24} is the weight of swollen film at a time "t = 24 h", W_0 is the weight of dried film at "t = 0".

2.4 Water vapor permeability

Water vapor transmission rate (WVTR) was determined gravimetrically using a modified ASTM Method E 96–95. The film specimen was mounted on an acrylic permeation cell comprised of two chambers. The upper chamber was in contact with water vapor pressure, while the bottom chamber was filled with an adsorbent material. The film specimen was in between both chambers, acting as a barrier. Therefore, the driving force of the global process was the difference in water vapor pressure at both sides of the film specimen. Once the permeation cell was assembled, all systems were placed into a chamber with temperature and relative humidity control. The operational conditions are fixed at 37 ± 2°C and 98% relative humidity (RH). Water vapor permeability (WVP) (ng·m·m^{-2}·s^{-1}·Pa^{-1}) was calculated from [11]:

$$\mathrm{WVP} = \frac{\mathrm{WVTR} \times l}{\Delta P} \tag{4}$$

where: WVTR ($ng \cdot m^{-2} \cdot s^{-1}$) was measured through a film specimen; l (m) was mean film thickness, ΔP (Pa) was partial water vapor pressure difference across the two sides of the film specimen.

2.5 Gas permeation

Flexible packaging materials must fulfill some specific characteristics according to the food they will pack. Fruits and vegetables are a particular type of food because they continue breathing after harvesting. Fruits and vegetables need oxygen to breathe, converting carbohydrates into carbon dioxide and water vapor. Post-harvest respiration uses stored starch or sugar and will stop when these reserves are exhausted. Therefore, designing a film that can retard fruits and vegetable respiration by controlling oxygen permeability and nitrogen and carbon dioxide exchange is desired. This condition might modify the atmosphere around the fruits and vegetables, altering oxygen levels inside the packaging, retarding the production of ethylene, and, thus, limiting the physiological decay of the product [12, 13]. This modification also reduces ripening-induced quality degradation in texture or loss of bioactive compounds during storage.

On the other hand, a minimal amount of oxygen might let anaerobic fermentation process, leading to spoilage [14]. For that reason, studying gas permeation through pectin and cross-linked pectin films is necessary to define the applicability of these films to the packaging of fruits and vegetables. In this study, N_2, O_2, and CO_2 permeability were measured at 30°C and 1 bar using a classical time lag apparatus. The effective membrane area was 11.34 cm^2, and permeate constant volume was 35.37 cm^3. After the membrane degassing procedure, gas permeation measurements were carried out under high vacuum ($p \approx 10$ torr) and 30°C for 10 h. The amount of gas transmitted at time "t" through the membrane was calculated from the permeate pressure (p_2) readings in the low-pressure side of the permeation cell. Permeability coefficients (P) were obtained from the flow rate into the downstream volume upon reaching the steady-state as:

$$P = \frac{Bl}{T_c p_1} \frac{\mathrm{d}p_2}{\mathrm{d}t} \tag{5}$$

where: the cell constant B = 11.53 (cm^3(STP) K)/(cm^2 cmHg); high-pressure side p_1 (cmHg); membrane thickness l(cm), the slope of the p_2 versus t plot in steady-state $\mathrm{d}p_2/\mathrm{d}t$ (cmHg/s), the temperature of the permeation cell T_c (K). Permeability values were obtained in Barrer unit (B), i.e., 1 $B = 10^{-10}$ cm^3 (STP) $cm \cdot cm^{-2} \cdot s^{-1} \cdot cmHg^{-1}$ and then converting Barrer to other units for comparison purposes.

Theoretical separation factors (α) were calculated from the relation between the permeation coefficients of pure $i$and$j$ gases as:

$$\alpha_{i/j} = \frac{P_i}{P_j} \tag{6}$$

3. Results and discussion

3.1 Mechanical properties

Mechanical properties of pectin and cross-linked pectin films were evaluated through strength-strain curves of each sample. Besides, commercial pectin was also analyzed. **Table 1** shows values of young's modulus (E; MPa), tension at break

Film	E(MPa)	σ(MPa)	ε(%)	T (kJ/m^3)
Pec	614 ± 8.9	12.6 ± 0.5	2.7 ± 0.3	15 ± 4
Pec-Ca	989 ± 10.1	21.3 ± 4.0	2.4 ± 1.0	265 ± 8
Pec-Fe	876 ± 9.8	22.0 ± 5.0	4.3 ± 1.0	511 ± 10
Com-Pec	176 ± 3.2	17.2 ± 1.7	30.3 ± 2.4	3330 ± 7

Table 1.
Mechanical properties of films.

(σ; MPa), elongation at break (ε; %), and tenacity (T; kJ·m^{-3}) of all samples. Results showed an increase in young's modulus and tension at the break with cross-linking, with the modulus being higher in the presence of calcium ions and retaining almost the same tension at break concerning Pec-Fe. An increase in elongation at break was observed in Pec-Fe, additionally with a higher energy absorption during the deformation process. Com-Pec resulted in a more deformable and resilient film reaching 30% of elongation with a tenacity one order of magnitude higher concerning the other samples. Mechanical results depicted a stiffness effect of cross-linking, more pronounced in Pec-Ca. This result might be because calcium ions better accommodate within pectin chains to form a stable egg box conformation regarding Fe ions. Although the ionic radius of calcium ions is higher than for iron ions, their divalent charge better interacts with two adjacent carboxylic acid units negatively charged within two entangled pectin chains. **Figure 1** shows the egg box model for Pec-Ca. Cybulska et al. [15] mentioned that the binding process in chain-to-chain pectin interactions with Ca^{2+} ions required a pronounced shift of one galacturonate chain concerning the other chain. Thus, the interaction of calcium ions with polygalacturonate chains may occur via oxygen atoms in the carboxylate group, in the ring, in the glycosidic bond, and the hydroxyl group of the next residue. Cross-linking formation at pH3.2 may be connected with the binding of calcium ions to pectin, hydrophobic interactions, and the formation of hydrogen bonds [16]. In this case, the interaction of Ca^{2+} with other oxygen atoms in galacturonic acid residues was suggested.

Furthermore, water molecules might also interact with ions, competing with carboxylic acid units to stabilize them. The calcium egg-box model is formed in a two-fold conformation of pectin chains. Remnant water molecules within the pectin matrix might provoke a polymorphic transition from two-fold to three-fold chain conformation, disrupting the egg-box configuration. This fact might explain results obtained by Pec-Fe, which showed lower young's modulus and higher elongations at break correlated with a more hydrated configuration. Regarding the structural possibilities of pectin-Fe, it could be like xanthan gum-Fe studied by Vazquez et al. [17].

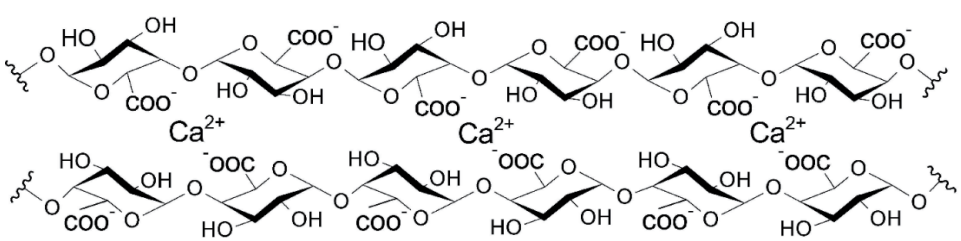

Figure 1.
Calcium "egg-box" model for pectin, based on [15].

3.2 Water uptake analysis

The hydrophilic nature of polysaccharides is a known characteristic that prevents some of these biopolymers' applications. Some strategies to reduce water uptake in films are cross-linking, blending, and mixing with other materials such as hydrophobic polymers [18], waxes [19], inorganic components [20], among others. In this study, cross-linking of the pectin matrix with calcium and iron ions was used to reduce the water uptake ability of film samples. **Table 2** shows WU results. A reduction in absorption capacity was observed in the presence of cross-linking concerning criolla orange pectin. Even when all values were significantly high, they depicted a weak cross-linking effect on water uptake. This result might be a consequence of the method used to cross-link the samples, which was ion diffusion from slightly concentrated solutions toward the matrix of the submerged film. Furthermore, this result might be evidence of superficial instead of full matrix cross-linking. It is well known that diffusion is a mass transport mechanism driven by concentration gradients and facilitated by temperature and stirring. The cross-linking procedure was performed under ambient temperature in stagnant conditions. Besides, Pec-Ca showed lower water absorption than Pec-Fe following its tighter egg-box cross-linking conformation mentioned in the previous section. On the other hand, Com-Pec showed complete solubility in water after a contact time of 24 h. This result might be explained considering the low methoxyl content of commercial pectin (galacturonic acid ≥74.0%, methoxy groups ≤6.7%) compared to criolla orange pectin [2]. Methoxy groups confer hydrophobic characteristics to the pectin backbone, which explains Pec's lower solubility in the water regarding Com-Pec.

3.3 Water vapor permeability

Permeability depends on the solubility and diffusivity of water vapor molecules within the polymeric matrix [11]. When the polymer is hydrophilic, water molecules find many interacting sites and hopping by the polymer matrix through the formation of hydrogen bonds [21]. Hence, water vapor permeation is facilitated within hydrophilic polymers such as pectin. Despite this disadvantage, cross-linking reduces these interactions by blocking polar groups on pectin such as carboxylate and hydroxyl groups through interactions with divalent and trivalent ions such as Ca^{2+} and Fe^{3+}. In order to study the effect of cross-linking on water vapor permeation, measurements were made gravimetrically using a modified ASTM Method E 96–95 (ASTM 96). Results are shown in **Figure 2**. A slight decrease in WVP of Pec-Ca concerning Pec was observed.

On the contrary, Pec-Fe showed an increase regarding orange pectin. This last result might be explained by an increased solubility of water vapor molecules through the polymer matrix favored by interactions with trivalent iron ions in the three folded chain conformation. Com-Pec showed the highest permeation value

Film	**WU(%)**
Pec	372 ± 38
Pec-Ca	326 ± 20
Pec-Fe	338 ± 25
Com-Pec	—

Table 2.
Water uptake values.

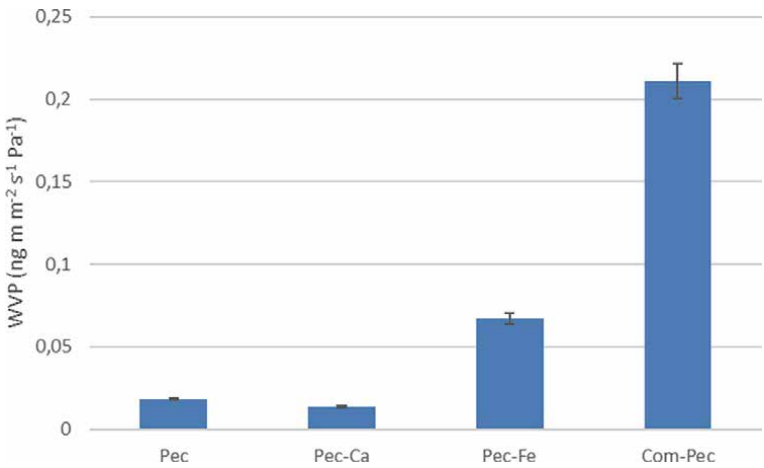

Figure 2.
Water vapor permeation of pectin films.

according to the hopping mechanism of water vapor molecules proposed by Cruces et al. [21].

As mentioned before, Com-Pec has ≥74.0% of de-esterified carboxylic acid groups; hence the polymeric matrix is full of polar groups able to interact with water vapor molecules to hop through. WVP result of commercial pectin agreed with that reported by Cruces et al. (0.0361 ng·m·m^{-2}·s^{-1}·Pa^{-1}) [21]. Other authors have studied water vapor permeation properties in polysaccharides and cross-linked polysaccharides. Values in the range from 1.5 to 0.6 ng·m·m^{-2}·s^{-1}·Pa^{-1} were reported for alginate-calcium cross-linked films [22], starch-based biopolymer with rye flour, cellulose, and citric acid as additives showed a WVP value of 0.87·ng·m·m^{-2}·s^{-1}·Pa^{-1} [23], xylan-alginate films containing bentonite, or halloysite clays showed a reduction in WVP from 0.394 for control film to 0.210 for 5 wt% for either clay [24]. Considering the reported WVP values for a variety of biopolymers, it is concluded that sensitivity to water vapor of hydrophilic polymers is still a matter of study.

3.4 Gas permeation

Gas permeation was measured in pectin films to analyze their ability to control gas exchange between internal and external sides of the packaging. According to our knowledge, O_2 and CO_2 are among the most important gases that take part in fruits and vegetable respiration. N_2 is an inert gas representing about 78% of atmosphere content, and it might show preservation effects [25]. The three main gases used in modified atmosphere packaging (MAP) are N_2, O_2, and CO_2. Decreasing the respiratory rate of fruits and vegetables in food packaging retards their deterioration. This effect occurs by reducing at least 5% of O_2 permeability, heightening CO_2 concentration, and regulating N_2 exchange inside the packaging. Oxygen promotes several deteriorative reactions in food, such as fat oxidation, browning reactions, and pigment oxidation. Besides, oxygen is necessary for bacteria and fungi growth. Carbon dioxide dissolves readily in water, increasing the acidity of food surroundings which can cause pack collapse due to the reduction of headspace volume. Nitrogen does not support the growth of aerobic microbes, and it is used to balance the volume decrease caused by CO_2 solubilization in water [26]. Gas permeation results and gas selectivity are shown in **Tables 3** and **4**, respectively.

Film	Thickness (μm)	P_{N2}	P_{O2}	P_{CO2}
Pec	152	3.87	1.44	0.33
Pec-Ca	90	3.34	2.84	0.40
Pec-Fe	258	5.16	1.45	0.59
Com-Pec	254	3.06	0.49	2.04

P = $(cm^3 \cdot \mu m \cdot m^{-2} \cdot d^{-1} \cdot atm^{-1})$.

Table 3.
Gas permeation in pectin films.

Film	$\alpha_{N2/O2}$	$\alpha_{O2/CO2}$	$\alpha_{N2/CO2}$
Pec	2.68	4.40	11.78
Pec-Ca	1.17	7.09	8.33
Pec-Fe	3.56	2.46	8.73
Com-Pec	6.21	0.24	1.50

Table 4.
Gas selectivity.

Results shown in **Table 3** depict a modified atmosphere by pectin and cross-linked pectin films. Considering fruit or vegetable packed in these films, it would be possible to see that normal atmosphere content (78% N_2, 21% O_2, and 0.01% CO_2) and its gas ratios have been modified. These results are better observed from **Table 4**, which we analyzed forwards. From **Table 3**, a reduction in N_2 permeability can be observed in the case of Pec-Ca and Com-Pec regarding orange pectin. On the contrary, an increase in P_{N2} was observed for Pec-Fe. Oxygen permeability increased for Pec-Ca, demonstrating a detriment in its ability to reduce oxygen content inside the packaging.

On the other hand, P_{CO2} was lower in the case of Pec, Pec-Ca, and Pec-Fe than Com-Pec. The barrier to CO_2 might represent a promising property for MAP. Differences in gas permeability between calcium and iron cross-linked films might be related to the polarity of ions concerning gases. Besides, the availability of ions within the less hydrated two-folded chains in the case of Ca^{2+} or more hydrated three-folded chains conformation in Fe^{3+} could also influence the interactions with permeate gases. These molecular conformations can also explain the increment in P_{CO2} for Pec-Fe. **Table 4** shows gas selectivity for selected gas pairs taking into account their abundance and gas ratio in the usual atmosphere. N_2/O_2 ratio in a usual atmosphere is around 3.71. From **Table 4**, it is observed that Pec-Fe has the closest value to that of the familiar atmosphere, while Pec and Pec-Ca have lower ratios and Com-Pec has the highest one. These results indicate that all films act as selective gas barriers favoring the permeance of N_2 more than O_2, except for Pec-Ca, in which N_2/O_2 selectivity is almost 1, i.e., no selectivity for N_2 nor O_2.

On the other hand, selectivity to O_2 against CO_2 was pronounced in the Pec-Ca film, followed by Pec and Pec-Fe. These results prove that an excellent barrier to CO_2 is reached in cross-linked films. Furthermore, Com-Pec showed an opposite behavior concerning O_2/CO_2 selectivity being more permeable to CO_2. Finally, N_2/CO_2 selectivity was excellent for Pec, and it was similar in the case of Pec-Ca and Pec-Fe. Values shown in **Table 4** indicate that N_2 permeability can balance the volume decrease caused by CO_2 solubilization in water as respiration and transpiration occur in fruits and vegetables. Commercial pectin showed less selective films for N_2/CO_2 gas pair according to its lower barrier to CO_2.

According to Sandhya [26], there has been much commercial interest in developing films with high gas transmission rates. High gas transmission films are obtained by modifying the film manufacturing process so that gases such as O_2, CO_2, and water vapor exit or enter the package in a controlled manner such that aerobic respiration needs are met, and desirable CO_2 and moisture levels are maintained. This work successfully controlled gas permeation and selectivity to obtain a modifying atmosphere inside packaging were achieved.

4. Conclusions

An exhaustive analysis of the effect of cross-linking of the pectin matrix with calcium and iron ions was carried out. Mechanical and permeation properties were studied and discussed to find new insights about structure–property relationships of modified films. Mechanical stiffness was observed when pectin was cross-linked with Ca^{2+} and Fe^{3+} showing higher Young's modulus and tension at break than orange and commercial pectin. Reduced water uptake was observed for Pec-Ca and Pec-Fe. However, values still being high concerning synthetic polymers. Similar results were obtained for water vapor permeation being Pec-Ca which depicted the lowest value. Finally, gas permeation assays were performed, demonstrating a good ability of cross-linked films to modify the atmosphere inside a packaging destined for fruits and vegetables packaging.

As a general conclusion, the conformation of two-folded chains in Pec-Ca concerning the three-folded chains in Pec-Fe was responsible for obtaining stronger mechanical properties, lower water uptake and water vapor permeation, and promising O_2/CO_2 selectivity in this cross-linked film.

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Pectin is an industrial product of certain fruit peels that contain it, such as citrus fruits, apples, pears, grapes, plums, beets, sunflowers, and so on. It is the traditional gelling agent for jams and jellies, but its applications extend to fruit products for food, dairy, dessert, soft drink, pharmaceutical, and other industries. This book discusses pectin production, pectin biotechnology, and pectin applications. Chapters cover such topics as the production of pectin from citrus, fungal pectinases in food technology, pharmaceutical applications of pectin, and more.

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