DEVELOPMENT OF PECTIN-WAX COMPOSITE EDIBLE FILMS WITH HIGH MOISTURE BARRIER PROPERTIES

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We approve the thesis of **Gamze ÇAKITLI**

Examining Committee Members:

Prof. Dr. Ahmet YEMENİCİOĞLU
Department of Food Engineering, İzmir Institute of Technology

Assoc. Prof. Dr. Beste BAYRAMOĞLU
Department of Food Engineering, İzmir Institute of Technology

Prof. Dr. Oğuz BAYRAKTAR
Department of Bioengineering, Ege University

3 July 2024

Prof. Dr. Ahmet YEMENİCİOĞLU
Supervisor, Department of Food Engineering,
İzmir Institute of Technology

Assoc. Prof. Dr. Ayşe Handan BAYSAL
Head of the Department of
Food Engineering

Prof. Dr. Mehtap Eanes
Dean of the Graduate School of
Engineering and Science

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ABSTRACT

DEVELOPMENT OF PECTIN-WAX COMPOSITE EDIBLE FILMS WITH HIGH MOISTURE BARRIER PROPERTIES

This thesis aimed development of multipurpose citrus pectin-based edible films showing moisture barrier and antimicrobial properties at the same time. For this purpose, pectin was composited with different waxes and antimicrobial essential oil carvacrol employing emulsification with hot homogenization. Pectin composite films with 15% glycerol and 40% beeswax obtained with $CaCl_2$ cross-linking showed WVP of 1.58 g.mm/m^2.day.kPa (54% lower than WVP of pristine pectin film). In contrast, pectin films with 15% glycerol and 30% carnauba wax showed a dramatically low WVP of 0.16 g.mm/m^2.day.kPa without $CaCl_2$ cross-linking (95% lower than WVP of pristine pectin film). These findings clearly showed that use of a wax with high melting point (m.p. of carnauba wax=82-86^oC) is more effective than one with low melting point (m.p. of carnauba wax=62-66^oC) to achieve composites with high moisture barrier properties. Therefore, antimicrobial films were developed by incorporating carvacrol at 1, 2, or 3% into pectin-carnauba wax composite films. The carvacrol incorporated edible composite films showed antimicrobial activity on *Escherichia coli* and *Listeria innocua* at a concentration dependent manner, but the increase of essential oil concentration reduced the moisture barrier properties of films. As a result, the antimicrobial pectin-carnauba wax composite films incorporated with 1% of carvacrol showed the highest moisture barrier properties with a WVP of 1.47 g.mm/m^2.day.kPa (57% lower than WVP of pristine pectin film). P). The compositing with waxes reduced the transparency and mechanical strength of pectin-based films. However, this thesis showed possibility of reducing moisture loss and microbial load of food by pectin-based packaging.

ÖZET

YÜKSEK NEM BARİYER ÖZELLİĞİ OLAN PEKTİN-MUM KOMPOZİT YENİLEBİLİR FİLMLER GELİŞTİRİLMESİ

Bu tez çalışmasında literatürde ilk kez turunçgil pektininin aynı anda hem su buharı geçirgenliği (WVP) düşük, hem de antimikrobiyel etki gösteren çift fonksiyonlu filmlerinin üretilmesi amaçlanmıştır. Bu amaçla ulaşmak için pektinin mumlarla sıcak homojenizasyon yöntemiyle emülsifikasyon yöntemi kullanılarak karvakrol içeren kompozit filmleri üretilmiştir. Pektinin %15 gliserol ve %40 balmumu içeren kompozit filmleri CaCl2'le çapraz bağlandıktan sonra elde edilen en düşük WVP değeri 1.58 g.mm/m^2.gün.kPa olarak belirlenmiştir (kontrol pektin filme göre %54 daha düşük WVP). Buna karşın pektinin %15 gliserol ve %30 karnauba mumu kullanılarak elde edilmiş filmleri çapraz bağlama uygulanmadan 0.16 g.mm/m^2.gün.kPa gibi çok düşük bir WVP değeri göstermiştir (kontrol pektin filme göre %95.4 daha düşük WVP). Elde edilen bu sonuç karnauba mumu gibi erime noktası yüksek (e.n.=82-86 ^oC) mum kullanımının erime noktası düşük (e.n.= 62-66 ^oC) bal mumu kullanımına göre WVP değerini daha etkili düşürdüğünü göstermiştir. Dolayısıyla antimikrobiyal film üretimi amacıyla karnauba mumu içeren pektin film kompozitleri içerisine %1, 2 veya 3 oranında karvakrol ilave edilmiştir. Elde edilen karvakrol içeren tüm filmler *Escherichia coli* ve *Listeria innocua* üzerinde esansiyel yağ konsantrasyonuyla orantılı olarak antimikrobiyal etki göstermiştir. Ancak, filmlerin WVP değerleri de ilave edilen esansiyel yağ konsantrasyonu arttıkça artmıştır. Bu nedenle tezde elde edilmiş olan en düşük WVP (1.47 g.mm/m^2.gün.kPa) gösteren antimikrobiyal film %1 oranında karvakrol içeren olmuştur (kontrol pektin filme göre %57 daha düşük WVP). Filmlere karnauba mumu ilavesi ışık geçirgenliklerini azaltırken mekaniki özelliklerini de kısmen zayıflatmaktadır. Bu tez çalışması gıdalarda daha az nem kaybına neden olabilecek antimikrobiyel pektin temelli film üretiminin mümkün olduğunu göstermiştir.

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CHAPTER 1

INTRODUCTION

The earliest record about use of small and large intestines of cattle and sheep for meat-stuffed casings (sausages) was discovered in almost 4,000-years-old Sumerian tablets found in Mesopotamia (Eckholm 1985). Thus, it could be accepted that edible packaging is an ancient technology that has been used more than 6000 thousand years to protect food products from physical and chemical quality changes and/or microbial contamination. In contrast, the use of moisture barrier edible packaging in food preservation is not too old as this was developed and applied first by Chinese using edible wax coatings for preservation of oranges in 12th century (Hardenburg 1967). Moreover, it is also thought that the first self-standing edible film is Yuba a proteic film developed by Japanese in 15th century by forming this film at the surface of boiled soy milk and applying it for wrapping of food (Umaraw and Verma 2017). In general, proteins, polysaccharides and waxes obtained from plant and animal sources have been heavily used for development of edible films, coatings and casings using classical methods such as solution-casting, dipping and spraying, and extrusion, respectively (Yemenicioglu 2022). However, hydrocolloids extracted from emerging and novel sources (e.g., bacterial, fungal and algal sources, insects, etc.) as well as the novel formulation and application techniques (e.g., nanoemulsions, nanocomposites, layer-by-layer coating, electrospinning, etc.) are a subject of great interest due to their promising potential to develop innovative food packaging systems (Sabina Galus et al. 2020; Yemenicioglu 2022). The major functionalities of edible films and coatings are as follows;

- Moisture barrier: Prevents dehydration, minimizes water vapour transmission, thus, extends shelf life (Kocira et al. 2021; Summo et al. 2022).
- Gas barrier: Edible films can control the permeability of gases such as oxygen, carbon dioxide, and ethylene. (Summo et al. 2022).
- Protection: protects food products from mechanical damage and physical changes (Bizymis and Tzia 2022).
- Maintenance of food quality: minimizes negative effects on texture, flavor, or color (Bizymis and Tzia 2022; Kocira et al. 2021).

- Preservation: edible film and coating helps to reduce the microbial contamination of food and to improve their shelf life and nutritional properties (Prasad et al. 2023).

Besides an individual polysaccharide, protein, or lipid, a combination of these film-forming materials (blends or composites) can also be used to create edible films and coatings (Abdollahzadeh et al. 2021). Therefore, mechanical, physicochemical and barrier properties of edible films might show a great variation depending on their composition. In general, protein based edible films show good oxygen barrier properties, but poor water vapour barrier properties. Polysaccharides are abundant materials mostly with excellent film-forming abilities, and good oxygen, aroma and lipid barrier effects, but most of them suffer from lack of water resistance and moisture barrier properties. Lipid-based edible films, especially beeswax and carnauba wax show excellent moisture barrier properties owing to their hydrophobic properties, but such lipid-based films show poor mechanical properties (Jeevahan and Chandrasekaran 2019; Falguera et al. 2011). Thus, to reduce disadvantages of individual edible film components different approaches have been developed such as enzymatic or chemical cross-linking or modification, blending or compositing with each other, use of nanoparticles as reinforcing fillers, and addition of surface active agents to improve emulsification (Garavand et al. 2017). Recently, a significant interest has been focused on compositing multiple film-forming agents to explore the complementary advantages of each component (Campos et al. 2011; Galus and Kadzi 2015). In most studies, compositing targets mainly improving mechanical characteristics or barrier properties of edible films (Bourtoom 2008). In particular, most composite films or coatings combine a hydrophilic hydrocolloid with a hydrophobic lipid component in order to improve moisture barrier properties of resulting films or coatings (Bosquez-Molina et al. 2003; Hassan et al. 2018). The emulsification is the primary method used to combine hydrocolloids with lipids (Galus and Kadzi 2015) but coating of a layer of hydrophilic edible film with a lipid layer (bi-layer films) is also a method to obtain moisture barrier edible films and coatings (Vieira et al. 2021). Edible films are not only capable to act as a moisture or gas barrier, but they can also act as active packaging by delivering incorporated or impregnated active compounds such as antioxidants and antimicrobials, flavour compounds, bioactive substances or nutrients onto food surface (Yemenicioglu 2022; Benbettaïeb et al. 2019; Tavassoli-Kafrani et al. 2016). The delivery of antimicrobials by edible films onto food surface named

“antimicrobial packaging” is the most frequently applied active packaging method since it enables inhibition of pathogenic or spoilage microorganisms at the food surface and improves food safety and shelf-life. The most frequently used antimicrobials in edible films include chemical antimicrobial food additives such as sorbic acid, benzoic acid, propionic acid, lactic acid, etc. while natural antimicrobials include nisin, lysozyme, polylysine, lactoferrin, phenolic extracts and their pure components, and essential oils and their pure components (Yemenicioglu 2022). Citrus pectin the major commercial pectin used worldwide is one of the most frequently used edible packaging and coating material since (1) it could be applied on to foods easily by dipping or spraying; (2) it could be used for delivery of both hydrophilic (e.g., ascorbic acid and derivatives, organic acids, etc.) and hydrophobic (e.g., essential oils) antimicrobials (Yemenicioglu 2022); and (3) its films show selective gas permeability (high selectivity ratio for CO_2/O_2 permeability) that is beneficial to extend the shelf-life of coated or packed fresh fruits and vegetables (Gontard et al. 1996). However, a major disadvantage of citrus pectin films is that they show poor moisture barrier properties. The main strategies employed to enhance the poor moisture barrier properties of pectin films involve cross-linking of films via divalent cations such as Ca^{++} (Çavdaroğlu et al. 2023a) and compositing of pectin with waxes by exploiting ability of pectin to form and stabilize emulsions (Alvarez-Perez et al. 2015).In the literature, extensive studies have been conducted to obtain antimicrobial pectin films (Ngo et al.2020; Jovanović et al. 2021;Aitboulahsen et al. 2020; Zhang et al. 2022; Nastasi et al. 2022; Aitboulahsen et al. 2020). Different studies have also been performed to improve the moisture barrier performances of pectin films by using compositing strategies with waxes (Berenice et al. 2015; Abdollahzadeh et al. 2021; Cagri, Ustunol, and Ryser 2004; Campos et al. 2011; Sánchez-Ortega et al. 2014; Szulc et al. 2020; Espitia et al. 2014). In contrast, studies related to development of pectin films showing both antimicrobial and moisture barrier properties are scarce. This thesis aimed development of multipurpose citrus pectin-based edible films showing moisture barrier and antimicrobial properties at the same time. For this purpose, pectin was composited with a low and high melting point waxes (carnauba wax with a m.p of 82-86°C and beeswax with a melting point of 62-66°C) and antimicrobial essential oil carvacrol employing emulsification with hot homogenization. This thesis is original in that it is one of the first attempts to develop moisture barrier antimicrobial edible pectin films employing a natural antimicrobial compound.

CHAPTER 2

LITERATURE REVIEW

2.1. Edible Films and Coatings

A novel approach to food preservation is antimicrobial edible packaging. This technology offers means to delay microbial growth, control moisture loss/gain, and gas exchange, thereby promoting food quality and shelf life (Suhag et al. 2020). An edible packaging might be applied in the form of a self-standing film used for producing a pouch or for wrapping of food, or as a coating applied at the food surface by dipping, brushing or spraying. Edible films are produced from biopolymers that are carbohydrates, proteins, lipids and combinations of these (Han 2014; Das et al. 2020; Maan et al. 2021).

Schematic representation of classification of edible coating formed materials are shown in Figure 2.1.

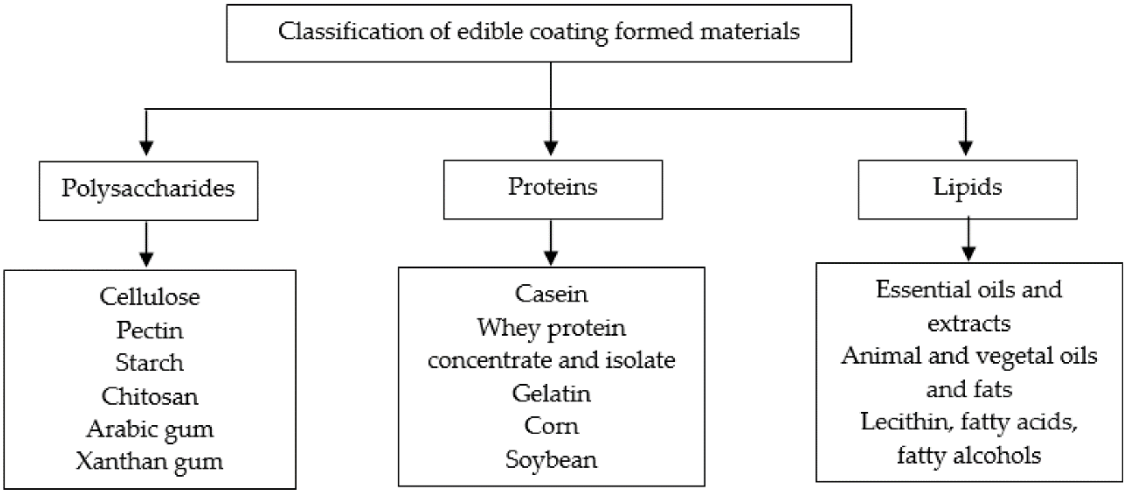

Figure 2.1. Schematic representation of classification of edible coating formed materials (Source: Nunes et al. 2023)

Edible films and coating made from polysaccharides such as cellulose, pectin, starch, chitosan, Arabic gum, xanthan gum or proteins such as casein, whey protein concentrate

and isolate, gelatine, corn zein, soybean proteins, animal and vegetal oils and fats used alone or as suitable blends and composites (Nunes et al. 2023; Gallo et al. 2000.)

The solution-casting method is the most frequenlty used method and consists of dissolving the biopolymer in a suitable solvent, ii) pouring the solution into the mold, iii) drying the poured solution (Figure2.2) (Suhag et al. 2020; Kumar et al. 2022).

a)

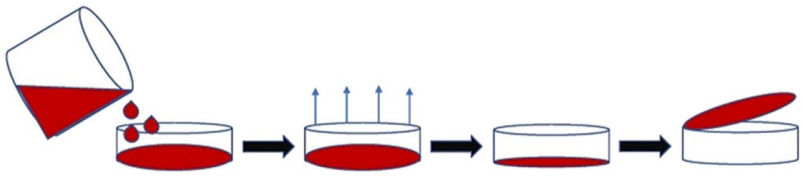

Figure 2.2. Edible film production by solution casting method (Source: Suhag et al. 2020)

Different methods could be used to apply edible coatings on fruits and vegetables (Figure 2.3). These methods involve dipping, spreading, spraying and wrapping (Pedreiro et al. 2021).

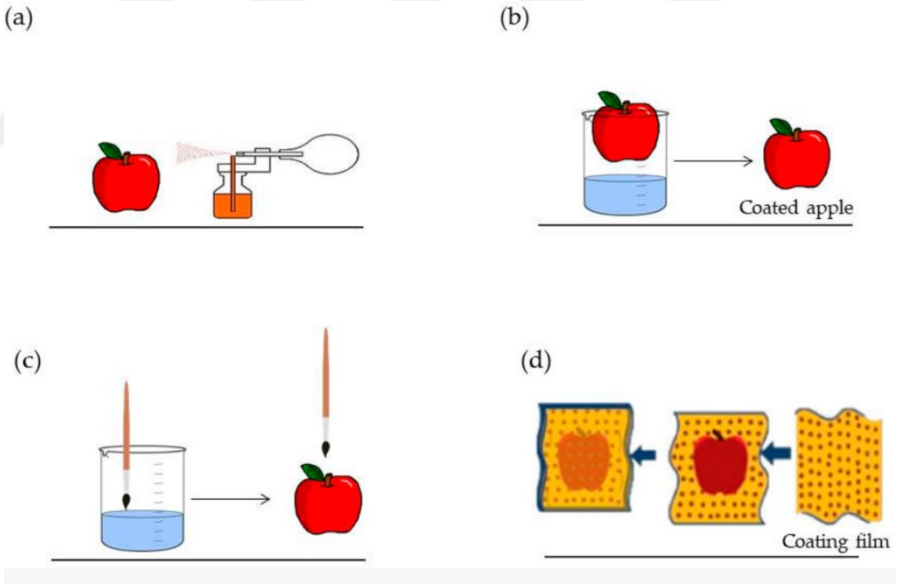

Figure 2.3. Schematic representation of coating techniques: (**a**) spraying; (**b**) dipping; (**c**) spreading and (**d**) wrapping (Source: Pedreiro et al. 2021)

The use of self-standing pectin films and coatings is limited due to their poor oxygen and water vapor barrier properties (Minh et al. 2020). Different strategies have been developed to improve the barrier properties of pectin films, but none of these studies find an effective solution applicable worldwide. There are different strategies to improve the physicochemical, functional and barrier properties of edible food packaging materials.

These include the cross-linking strategy and the addition of hydrophobic ingredients and fillers, including nanomaterials. A possible strategy to improve the barrier protperites of films is to chemically cross-link hydrocolloids forming the film matrix (Yao et al. 2022). Crosslinking method is used widely to improve the water vapour barrier property of film. It was reported that cross-linking agents cause an increase in the surface hydrophobicity of films, resulting in low water vapor permeability (Chambi and Grosso 2006). Divalent ions like Ca^{2+}, oxidized phenolic compound, organic acids, oxide particles and inorganic particles are used as crosslinking agents (Zhang et al. 2024). $Ca^{2+}$is frequently preferred for cross-linking process because it has advantages such as being a green method, availability and low cost (Zhang et al. 2024).

Li et al. (2016a) showed how $CaCl_2$ concentration affected the gelation process of alginate films (Figure 2.4) (Li et al. 2016a).

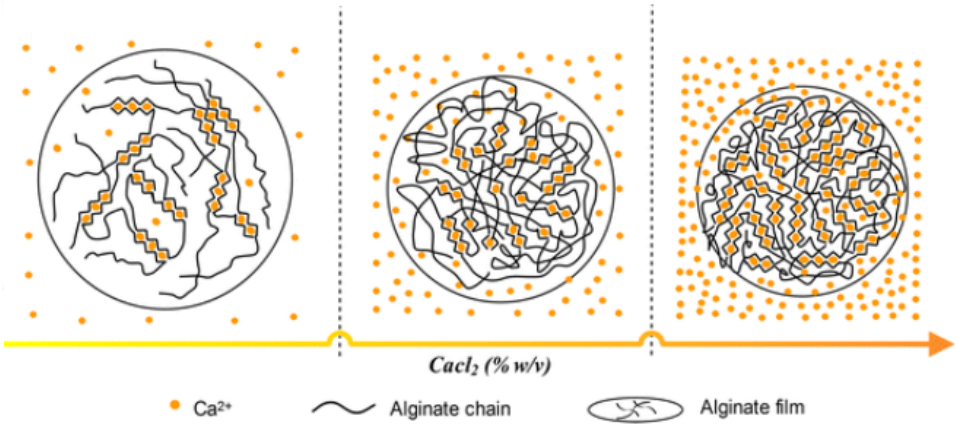

Figure 2.4. Schematic evolution of alginate gelation process with the increase of calcium chloride concentration (Source: Li et al. 2016b)

According to the literature, various substances such as antibacterial agents, antioxidants, nutrients, colorants, aroma compounds and spices could be incorporated into films to obtian different functins.

Edible films and coatings help to reduce oxidative chnages in food and to protect food from spoilage microorganism, and to reduce the risk of pathogenic microorganism growth in foods (Lisitsyn et al. 2021; Sánchez-Ortega et al. 2014; Ganiari et al. 2017). Developing techniques to extend the shelf life of products such as cheese, dried fruit and meat has been an area of research in recent years (Yuan et al. 2022; Ünalan et al. 2013). Antimicrobial edible films and coatings are seen as a promising preservation technology also for raw and processed meats against spoilage and pathogenic microorganisms

(Sánchez-Ortega et al. 2014). Antimicrobial agents including organic acids, bacteriocins, essential oils and phenolic extracts widely incorporated in edible films.

Ünalan et al. reported that lysozyme containing zein and zein-carnauba wax composite films suppressed the growth of *Listeria monocytogenes* in fresh cheeses during cold storage (Ünalan et al. 2013).

Fruit and vegetable juices or purees have been also used to produce edible films and add value to edible films because of their nutritional and antimicrobial properties (Yıldırım-Yalçın et al. 2021). It was prooved that edible film from mixture of grape juice, maize starch cross-linked with sodium trimetaphosphate and glycerol reduced lipid oxidation and microbial growth of food during cold storage (Yıldırım-Yalçın et al. 2021).

Recently, essential oils has been used in antimicrobial packaging extensively. The essential oils do not contain fatty acids as animal and vegetable oils, but they are highly complex volatile compounds (with up to 60 components) soluble in organic solvents containing several major (e.g., terpenes, terpenoids, and aromatic compounds) and many minor fractions (Bakkali et al. 2018). Essential oils interact with the lipid components of cell membranes, altering membrane permeability and causing microbial cell leakage. Carvacrol is one of the essential oils highly effective on both Gram-positive and Gram-negative bacteria (Yuan et al. 2022).

The brittleness in a also a major problem for most edible films. Therefore, plasticizers are frequently employed to obtian flexible edible iflms. However, high levels of plasticizer increases the water vapour permeability of the films. Therefore, optimizing the plasticizer concentration is crucial to produce edible film with desired flexibility and moisture barrier properties (Yıldırım-Yalçın et al. 2021). It has been reported that substances used as plasticizers in edible film formulation, such as glycerol, require the use on 10-60% on the dry matter basis, but such amounts affect water vapour and gas permeability negatively (Sothornvit and Krochta 2005).

2.1.1. Protein-Based Edible Films

Proteins are good film-forming substances that exhibit outstanding O_2, CO_2 and oil barrier properties but mechanical properties of protein films are weak especially under low relative humidity conditions (Cheng et al. 2023; Milani and Tirgarian 2020). Moreover

due to their hydrophilic structure, they have weak water vapor barrier properties (Milani and Tirgarian 2020).

By incorporating hydrophobic substances into protein matrices, the weak moisture barrier properties of single protein-based edible films can be overcome (Galus et al. 2020).

2.1.2. Lipid-Based Edible Films

Various waxes such as beeswax, carnauba wax, candle wax, soy wax and resins such as shellac resin, coumarin resin, turpentine are often used as lipid coating components (Wu, Wu, and Hu 2024).

Lipid materials are preferred in edible film production due to their hydrophobicity that increase water-resistance and moisture barrier properties of films (Milani and Nemati 2022).

Lipids are among the most preferred polymers because they provide hydrophobicity in edible film formulation, providing an excellent barrier feature against moisture and oxygen. Although lipid films have perfect barrier properties, they show poor mechanical properties, thus, should be combined with different polysaccharides and proteins to obtian self-standing films (Wu, Wu, and Hu 2024).

Among all natural waxes, beeswax and carnauba wax are the most studied (Yi Zhang, Simpson, and Dumont 2018).

Beeswax is a hydrophobic agent composed of a mixture of esters, hydrocarbons, fatty acids, alcohol, and others (Pérez-Vergara et al. 2020). It is considered a GRAS substance (generally recognized as safe) by the U.S. Food and Drug Administration (Szulc et al. 2020). Beeswax has been preferred in edible film formulation because it produces materials with greater resistance to water vapor, thanks to its long-chain fatty acid composition (Cort Es-Rodríguez et al. 2017). Incorporation of beeswax in film matrix has significant improvement in terms of moisture barrier properties (Diyana et al. 2021; Pérez-Vergara et al.2020; Haq et al.2016; Cheng et al.2023). Carnauba wax is a wax having one of the the greatest melting points. It consists of aliphatic esters and diesters of cinnamic acid and it is one of the hardest waxes in solid fiorm (Galus et al. 2020). It is yellowish and has a melting temperature around 82–86 °C Filho et al. 2020).

2.1.3. Polysaccharide-Based Edible Films

Polysaccharides including starch, chitosan, cellulose, pectin, alginate and seaweed and pulluan have been used for edible films (Kumar Dubey and Dubey 2020). Some of these polysaccharides, alginate, chitosan, pectin, and starch, are the most available and commonly used in the food industry (Cerqueira et al. 2023a). In the current study, pectin has been used as an edible film-forming material.

2.1.3.1. Pectin

Pectin, a major food hydrocolloid that is highly demanded by the food industry primarily as a gelling and thickening agent, is commercially produced from citrus peels (~85%). However, the increased applications of pectin in food as well as recent trends to valorize different agro-industrial wastes have encouraged industry to seek alternative pectin sources such as sun-flower head residues and sugar beet pulp that contain 10-20% pectin (d.w.) (Gawkowska et al. 2018). The pectin, the most complex polysaccharide in plants, is formed by three different fractions; homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II). The HG, RG-I and RG-II are attached covalently to each other (Harding et al. 2017) and form almost 65, 20-35 and 10% of pectin molecule in plants, respectively (Chandrayan 2018). The linear HG fraction forms the "smooth regions" of pectin molecule while branched RG-I and RG-II forms the "hairy regions". However, some other minor pectic fractions also exist such as xylogalacturonan, apiogalacturonan, arabinan, galactan, arabinogalactan I and arabinogalactan II (Gawkowska et al. 2018).

The HG is the major pectin fraction found in primary cell walls and middle lamella of plant cells, and it consists of linear chains of 1,4-linked α-D-galacturonic acid units (Watkins 2017). The carboxyl groups at C6 of galacturonic acid residues of pectin exist in free or methyl esterified form. Therefore, International Pectin Producers Association (IPPA) classifies pectin depending on DE (high methoxyl pectin: DE> 50%; low methoxyl pectin: DE< 50%).

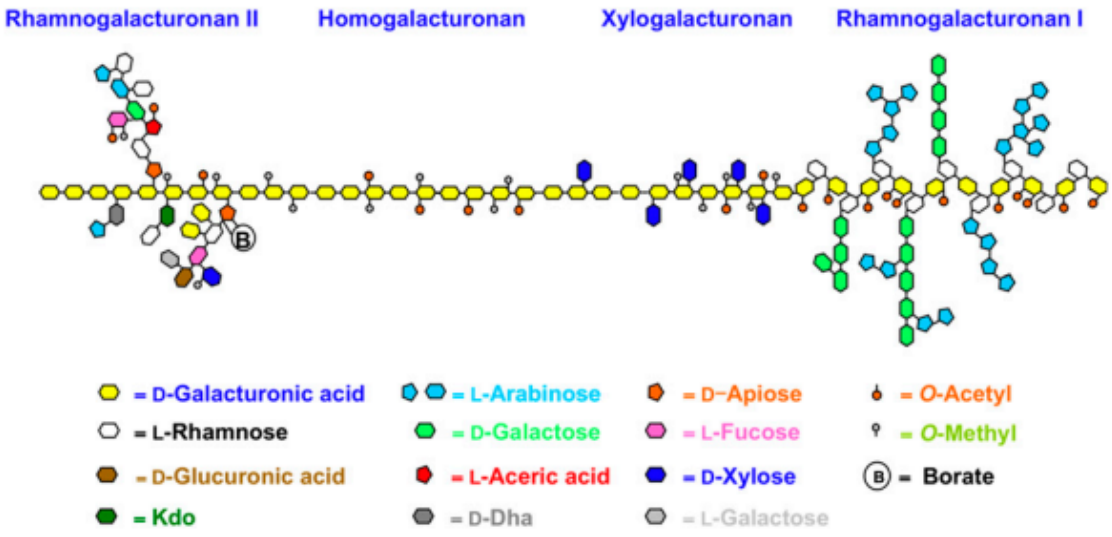

Figure 2.5. Schematic representation of pectin structure with its main components (Source: Harholt, Suttangkakul, and Scheller 2010)

Pectin is commonly utilized in the food industry due to its unique techno-functinal properties as an emulsifier, thickening, gelling agent, and stabilizer and also provides an important source of dietary fiber, which may have therapeutic properties (Lara-Espinoza et al. 2018; Normand et al. 2021).

Pectin's ability of pectin to serve several purposes is attributed to the molecule's unique composition affected from source of extraciton and extraction methods (Roman-Benn et al. 2023). In addition, it attracts the attention of researchers with its high biodegradability, insolubility in non-polar solvents, high solubility in water, non-toxicity and ability to form tasteless, odorless and colorless forms (Cerqueira et al. 2023b).

Pectins can be utilized to produce film/coating matrices either alone or with other compatible polymeric components (Lazaridou and Biliaderis 2020). Due to poor oxygen and water vapor barrier properties, industrial use of pectin films and coatings is limited (Minh et al. 2020). The objective of this study was development of multipurpose citrus pectin-based edible films showing moisture barrier and antimicrobial properties at the same time.

CHAPTER 3

MATERIALS and METHODS

3.1. Materials

Citrus pectin was purchased from Sigma Aldrich Co., Ltd (İzmir, Turkey). Glycerol and Tween 80 (hydrophilic/lipophilic balance, HLB = 15.0) were purchased from Merck (Darmstadt, Germany). *Listeria innocua* NRRL-B 33314 (ATCC 1915) and *Escherichia coli* RSHM 4024 (ATCC 25922) were from culture collection of the microbiology laboratory of the Department of Food Engineering at Izmir Institute of Technology. All chemicals used in the study were analytical grade unless otherwise mentioned and purchased from Sigma Aldrich Co., Ltd. (İzmir, Turkey).

3.2. Methods

3.2.1. Film preparation

3.2.1.1. Control Pectin

For this purpose, 3% (w/w) pectin solution was prepared and heated with constant stirring until it reached 60°C. After the solution was cooled to room temperature, it was treated at 10,000 rpm for 1 min using a homogenizer (Heidolph, Germany, rotor ϕ = 6.6 mm Tip). Then %30 (w/w of pectin) or 15% (w/w of pectin) was added as a plasticizer and the mixture was mixed for 15 minutes. Finally, the film solution was homogenized at 10,000 rpm for 4 minutes, and 10 g of the film solution was poured into a glass petri dish (inner diameter 10 cm) and dried in a controlled test cabinet at 25°C and 50% RH for 24 hours.

10 g portions of film-forming solution were poured into petri dishes (10 cm in diameter), and the dishes were dried at 25°C and %50 RH for 24 h. The films peeled-off from the Petri dishes were used in different analyses. The cross-linked films were obtained by treating dried films with 3% (w/w) $CaCl_2$ solution and drying films again in the controlled test cabinet at 25°C and 50% RH for 24 h. All pectin films were prepared in duplicate.

3.2.1.2. Preparation of Pectin-Bees Wax Composite Film

3% (w/w) pectin solution was prepared and heated with constant stirring until it reached 90°C. After reaching the desired temperature, the pectin solution was homogenized at 10,000 or 20,000 rpm for 1 minute. Then, 40% or 30% (w/w of pectin) beeswax, 15% or 30% (w/w of pectin) glycerol and 1% (w/v) tween 80 pectin solution were added. It was then left in a water bath at 90 degrees for 30 minutes. The film solution was homogenized for 4 minutes at 10,000 or 20,000 rpm and 10 g of the film solution was poured into a glass petri dish (inner diameter 10 cm) and dried for 24 hours at 25°C and 50% RH in a controlled test cabin. The cross-linked films were obtained by treating dried films with 3% (w/w) $CaCl_2$ solution and drying films again in the controlled test cabinet at 25 °C and 50% RH for 24 h. All pectin films were prepared in duplicate.

3.2.1.3. Preparation of Pectin-Carnauba Wax Composite Film

3% (w/w) pectin solution was prepared, but this time it was heated to 95°C due to the high melting point of the carnauba wax. After reaching the desired temperature, the pectin solution was homogenized at 10,000 rpm for 1 minute. Then, 30% (w/w of pectin) or 20% (w/w of pectin) carnauba wax was added and heated at 95°C for 30 min. Then, 15% (w/w of pectin) glycerol and 1% (w/v) tween-80 were added. Then it was mixed at 95°C for 15 minutes.

After mixing at 95°C for 15 minutes, for control carnauba-pectin composite films; the mixture was homogenized at 10,000 rpm for 7 minutes, for carnauba-pectin films with carvacrol; the mixture was homogenized at 10,000 rpm for 2.5 minutes, then 1%, 2% or 3% (w/v) carvacrol was added, homogenization continued same setting for 4.5 minutes.

After homogenization 1 minute mixing was done and 10 g of the film solution was poured into a glass petri dish (inner diameter 10 cm) and dried in a controlled test cabinet at 25°C, 50% RH for 24 hours.

Table 3.1. Different ratios of components for preparing films and their symbols in the current research

GLY (%w/w)	CW (w/w)	BW (w/w)	CAR (%w/v)	$CaCl_2$ (3% v/v)	Film symbol
15					PE-GLY15
30					PE-GLY30
15		30			PE-BW30-GLY15
		40			PE-BW40-GLY15
		30		1%	PE-BW30-GLY15-Ca++
		40		1%	PE-BW40-GLY15-Ca++
30		30			PE-BW30-GLY30
		40			PE-BW40-GLY30
		30		1%	PE-BW30-GLY30-Ca++
		40		1%	PE-BW40-GLY30-Ca++
15	20				PE-CW20-GLY15
	30				PE-CW30-GLY15
	30		1%		PE-CW30-GLY15-CAR1%
	30		2%		PE-CW30-GLY15-CAR2%
	30		3%		PE-CW30-GLY15-CAR3%

3.2.2. Film Characterization

3.2.2.1. Water Vapour Permeability of Films

WVP of pectin films was measured according to ASTM Standard Method E96 (ASTM, 2016) using Payne permeability cups (Elcometer 5100, UK). (ASTM, 2016). A film (diameter: 6 cm) and an O-ring were placed on top of each cup containing 3 g of dried silica beads, and the cups were closed with a metal ring with three screw clamps.

The cups were weighed and placed in a controlled test cabinet (TK 120, Nüve, Turkey) at 25°C and 50% relative humidity. The cups were weighed periodically over 72 hours and the measured weights were plotted versus time. To calculate WVP according to the equation 1, the linear portion of the curve with at least five data points was used ($R^2 \geq 0.99$), where G is the weight change from the straight line (g), L is the thickness of the film (mm), t is the time (day), A is the test area (m^2), S is the saturation vapor pressure at test temperature (3.169 kPa at 25°C, R_1 the relative humidity of the test chamber (50%) and R_2 the relative humidity in the dish (0%). Four independent tests per film were performed.

$$WVP = \frac{GL}{A_t\, S(R_1 - R_2)} \quad (1)$$

3.2.2.2. Film Solubility

The solubility of films were determined according to the method described by Pérez et al. 2016. Films (15 mm × 7.5 mm) were dried in a vacuum oven (Barnstead Lab-Line, Model 3608-6CE, USA) at 70°C for 24 h and weighed. Film pieces were placed in a test tube containing 10 mL of distilled water, and then were shaken with an orbital shaker (IKA, OS 5 Basic, Germany) at 240 rpm for 24 h and placed in an incubator at 25°C and %50 RH. The solids remaining in the tube were filtered and the insoluble dry matter content is collected and dried to constant weight with hot air at 105°C. Eight pieces of each film were tested. Membrane solubility (%) was determined from the equation 2.

$$(\%) = 100 * \frac{(Initial\ dry\ matter - Insoluble\ dry\ matter)}{Initial\ dry\ matter} \quad (2)$$

3.2.2.3. Morphological Properties of Films by AFM and SEM

The surface topographical images of the films were obtained by atomic force microscopy (AFM, MMSPM Nanoscope 8 from Bruker, USA) in an intermittent-contact mode in the air with silicon tips (resonance frequency approximately 340 kHz, spring constant approximately 40 N m^{-1}, tip radius 8 nanometer). Captured images (at least 4 per

sample) were analyzed using Nanscope Analysis software v.1.5 (Bruker, USA). The surface and cross-sectional morphology of the pectin films were examined using scanning electron microscope (SEM, 250 Quanta FEG, FEI Corporation, USA). The films were first freeze-dried and then placed into liquid nitrogen and crashed for SEM examination. The samples were then gold-coated using a sputter coater (Emitech K550X, Quorum Technologies Inc., UK) at 10 mA for 60 s.

3.2.2.4. Transparency

Film transparency was determined according to ASTM 2002b with modifications of the method described by Pérez et al. (2016). The transparency of the films was measured using a spectrophotometer at 600 nm. A rectangular piece of film (30 × 10 mm^2) was placed in the spectrophotometer cell and measured against the empty cell as a blank. Each film was tested in eight replicates. Transparency was calculated using equation 3 where b is the film thickness (mm).

$$T_{600}=\frac{(\log \%T)}{b} \quad (3)$$

3.2.2.5. Mechanical Properties of Films

Using a Texture Analyzer TA-XT2 (Stable Microsystems, Godalming, UK), the tensile strength at break (TS), elongation at break (EAB), and Young's modulus (YM) of films were determined according to ASTM Standard Method D 882–02 (ASTM 2002a). The films were dried in a controlled test cabinet at 25°C and 50% relative humidity (RH) for 24 h before testing. Then, the films were cut into 50 mm long and 8 mm wide strips. The initial grip distance was 50 mm, and the drawing speed was 50 mm min^{-1}. Minimum eight strips of each film were tested. The thickness of films was measured by using a micrometer (Chronos, UK).

3.2.2.6. Test of Film Antimicrobial Activity

Antimicrobial activity tests were performed using *Listeria innocua* (ATCC 1915) and *Escherichia coli* (ATCC 25922) as test microorganisms. The overnight cultures were prepared in nutrient broth and all incubations were conducted at 37°C. For antimicrobial testing, film discs (diameter: 1.3 cm) were prepared using a cork borer drill under sterile conditions. Antimicrobial activity test was conducted as two replicates. Twelve discs (2 disc was placed per Petri dish) from each film were tested at each replicates. The discs were placed carefully onto petri dishes containing nutrient agar on which 0.1 mL culture was spread. The petri dishes were incubated for 24 h at 37°C and the area of the fully formed zones observed was determined by measuring the zone diameter with a caliper.

3.2.2.7. Statistical Analysis

The data obtained from the analyses in triplicates and expressed as the mean value ± standard deviation. The statistical analysis was performed using Minitab (Version 18.1., Minitab Inc., United States). Statistically significance analysis was obtained using one-way ANOVA with Fisher's post hoc test with 95% confidence level ($P < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSIONS

This thesis aimed development of multipurpose citrus pectin-based edible films showing moisture barrier and antimicrobial properties at the same time. For this purpose, firstly pectin (PE) was composited with a low and high melting point waxes (carnauba wax (CW) with a m.p of 82-86ºC and beeswax (BW) with a melting point of 62-66ºC) to determine most successful moisture barrier films with lowest water vapour permeability (WVP) values.

4.1. Development of moisture barrier pectin films by compositing with beeswax

4.1.1. Water vapour permeability of pectin-beeswax composite films

4.1.1.1. Effect of beeswax and glycerol concentrations on water vapour permeability of films obtained without $CaCl_2$ cross-linking

During studies with PE-BW composite films, different concentrations of glycerol (GLY) (15% or 30%, w/w of PE) and BW (30% or 40%, w/w of PE) were tried to improve moisture barrier properties of films. The effects of BW and GLY concentrations on WVP of different composite films are shown in Figure 4.1. The results clearly showed that GLY concentration showed a remarkable effect on WVP of pristine PE films. For, example, reduction of GLY concentration of pristine PE films from 30% to 15% caused drop of WVP from 8.1 to 3.45 g mm/m^2.day.kPa. In contrast, the effect of BW concentration on WVP depend on GLY concentration of films. For films plasticized with 30% GLY, the WVP of 8.1 g mm/m^2.day.kPa for pristine PE film dropped to 4.6 and 4.3 g mm/m^2.day.kPa by addition of 30 and 40% BW, respectively. However, the addition of BW at 30 or 40% increased and did not affect the WVP of films plasticized with 15%

GLY, respectively. In fact, the pristine PE film plasticized with 15% GLY showed the lowest WVP of films obtained without $CaCl_2$ (3% w/v) cross-linking. This finding clearly proved that minimization GLY concentration is a more effective moisture barrier strategy than addition of BW without $CaCl_2$ (3% w/v) cross-linking.

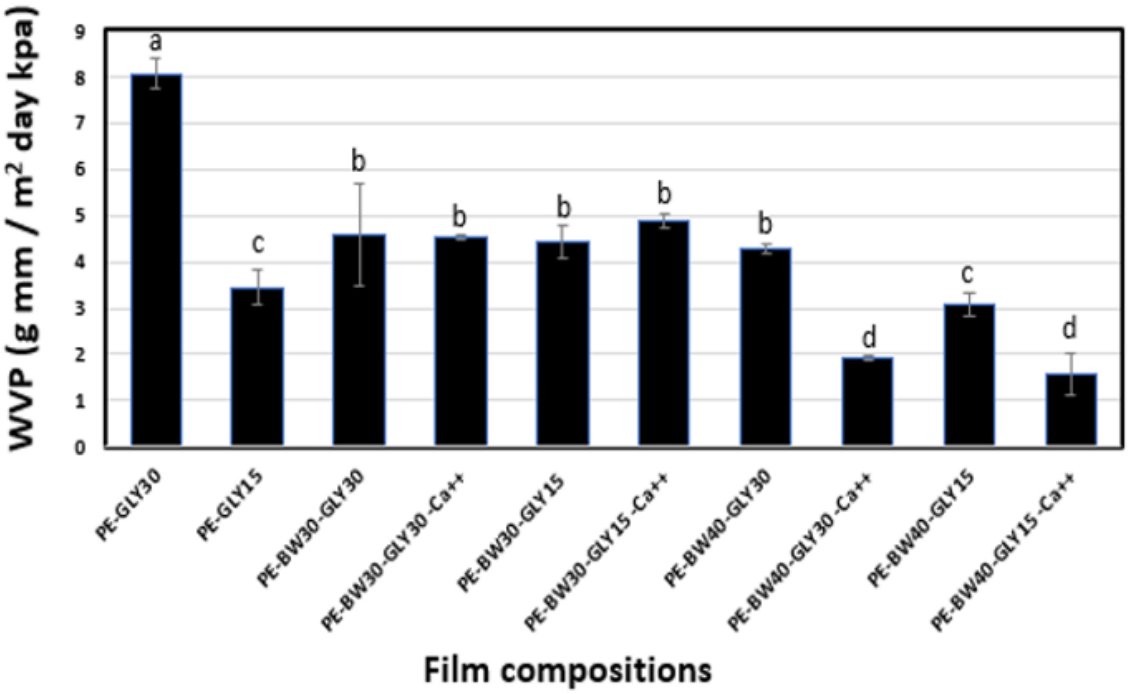

Figure 4.1. Effect of beeswax and glycerol concentrations and $CaCI_2$ induced cross-linking on water vapour permeabilities of different pectin-beeswax composite films (Different letters indicate significantly different values at $P < 0.05$).

4.1.1.2. Effect of beeswax and glycerol concentrations on water vapour permeability of films obtained with $CaCl_2$ cross-linking

The $CaCl_2$ cross-linking is another factor that could affect the WVP of developed films. However, is should be reported that this effect cannot be observed for pristine PE films as these films become highly brittle and could not be peeled off from petri dishes. In contrast, the PE-BW films did not turn brittle by $CaCl_2$ cross-linking, thus, allowed investigation of its affect in PE-BW composite films. It is noteworthy to note that the increase of BW content from 30 to 40% became a significant factor on WVP for films plasticized with 30% or 15% GLY when films were cross-linked with $CaCl_2$. In fact, the $CaCl_2$ cross-linked PE-BW films with 40% BW showed the lowest WVP without affecting from GLY content. These findings clearly showed that the improved structural

integrity of pectin film matrix by $CaCl_2$ cross-linking prevented the negative effects of plasticizer GLY on film WVP. Moreover, it is also clear that the tightening of film matrix with $CaCl_2$ cross-linking provided BW granules a more compact arrangement that show barrier effect on moisture.

4.1.2. Mechanical properties of pectin-beeswax composite films

The analysis of mechanical properties in edible films and coatings offers valuable insights into their anticipated durability. Furthermore, these properties can elucidate the films' and coatings' capacity to safeguard food products throughout the entire supply chain, from production to consumption (Arfat et al. 2021). The mechanical properties of films changed at a narrow range. The TS of films varied between 5.83 and 14.6 MPa while EAB values varied between 1.18 and 6.81%, and YM varied between 3.05 and 9.03 MPa (Table 4.1). The composite film-making with BW did not cause marginal changes in the mechanical properties of PE films. However, the lowest tensile strength was observed for PE-BW30-GLY15-Ca^{++} while the highest tensile strength was observed for PE-BW40-GLY15-Ca^{++}. Thus, it appeared that increased wax concentration resulted with an increase in TS of $CaCI_2$ cross-linked PE-BW films. Similarly, due to limited changes in film properties it is hard to inform marginal effects of GLY concentration and $CaCI_2$ induced cross-linking on mechanical properties of films. It is also important to report that films of PE-GLY15, PE-BW40-GLY15, PE-BW40-GLY15-Ca^{++} and PE-BW30-GLY15-Ca^{++} showed significantly higher Young's modulus (greater stiffness) than the other films. All these films are among those with 15% GLY. Thus, it is clear that the stiffness is a parameter highly affected from the plasticizer content. The first three of these films performed well in the WVP tests while the last film was not a good performer in WVP test. One of the good performers of WVP test was PE-BW40-GLY15-Ca^{++}, but this film gave a low Young's modulus value. Thus, it appeared that stiffness might be related with moisture barrier properties, but it is not the only factor determining the moisture barrier performance of films. Further studies are needed to compare other properties of films such as surface hydrophobicity and tortuosity.

Table 4.1. Effect of beeswax and glycerol concentrations and $CaCl_2$ induced cross-linking on mechanical properties of different pectin-bees wax composite films

Film Composition	GLY (w/w)	BW (w/w)	$CaCl_2$ (3%, w/v)	TS (MPa)	EAB(%)	YM (Mpa)
PE-GLY30	30%	-	-	10,86±1,14^{bcd}	4,56±2,13^{bc}	4,79±0,90^{c}
PE-GLY15	15%	-	-	12,43±2,68^{ab}	2,04±0,53^{ef}	8,55±0,20^{ab}
PE-BW30-GLY30	30%	30%	-	9,10±2,53^{de}	4,48±1,28^{bc}	3,84±1,41^{cd}
PE-BW30-GLY30-Ca^{++}	30%	30%	+	13,97±3,81^{a}	6,81±2,29^{a}	4,27±0,95^{cd}
PE-BW30-GLY15	15%	30%	-	7,83±1,63^{ef}	5,22±0,79^{b}	3,05±0,79^{d}
PE-BW30-GLY15 -Ca^{++}	15%	30%	+	5,83±3,77^{f}	1,18±0,41^{f}	7,25±1,92^{b}
PE-BW40-GLY30	30%	40%	-	7,17±1,90^{ef}	2,38±0,81^{def}	3,17±1,30^{d}
PE-BW40-GLY30 -Ca^{++}	30%	40%	+	9,40±3,44^{cde}	3,05±2,42^{cde}	4,50±0,97^{cd}
PE-BW40-GLY15	15%	40%	-	12,27±2,55^{abc}	1,63±0,44^{ef}	9,03±1,86^{a}
PE-BW40-GLY15 -Ca^{++}	15%	40%	+	14,60±2,31^{a}	3,71±1,02^{cd}	6,77±0,59^{b}

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

4.1.3. Film thicknesses and transparencies of pectin-beeswax composite films

The thicknesses of pristine PE films and PE-BW composite films are presented in Figure 4.2. Incorporation of beeswax (BW) resulted in a significant increase ($p < 0.05$) in film thickness. In fact, the thickness (µm) of pristine PE-GLY30 film with 30% glycerol increased from 33 µm to 73 µm and 61µm by addition of 30 or 40% beeswax wax, respectively. Similarly, the thickness (µm) pristine PE-GLY15 film increased from 33 µm to 81 µm and 66 µm by addition of 30 or 40% beeswax wax, respectively. The difference in GLY concentration did not affect thickness significantly ($p < 0.05$). Thus, it is clear that the packaging of wax particles within the films is highly effective on film thickness. These findings compare well with the literature as Syahida et al. (2020) also observed an increase in the thickness of Ca-pectate films with incorporation of palm wax.

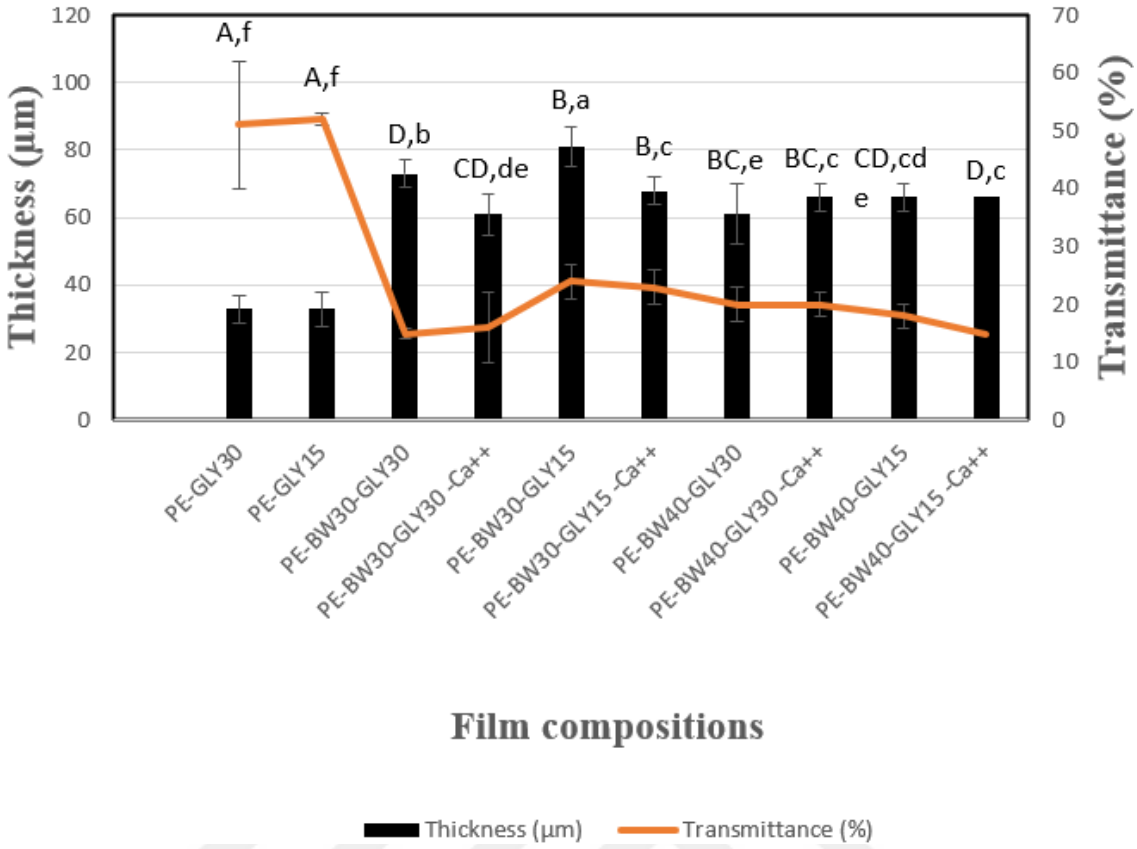

Figure 4.2: Effect of beeswax and glycerol concentrations and $CaCl_2$ induced cross-linking on thickness and transparency of different pectin-beeswax composite films. Different letters indicate significantly different values at $p < 0.05$. The capital letters are for values of transmittance values. Lower case letters are for thickness values).

4.1.4. Morphology of pectin-beeswax composite films in SEM and AFM

The SEM cross-sections of pristine PE films (PE-GLY15, PE-GLY15-Ca^{++} and selected PE-BW composite films, those performed well in WVP test (PE-BW40-GLY15, PE-BW40-GLY15 -Ca^{++} are seen in Fig. 4.8. The pristine PE films with or without $CaCl_2$ cross-linking showed a dense cross-section free from large voids, cracks and pores (Fig 4.8A and 4.8B). The PE-BW films also lacked cracks and large pores at cross-section, but numerous tiny wax droplets, and melt hairy branched wax aggregates were apparent at the cross-sections of films (Fig. 4.3C and 4.3D). The tiny micro-sized blisters (possibly wax particles embedded into films) were also observed partially at surfaces of films obtained with $CaCl_2$ cross-linking. It should also be noted that the PE-BW40-GLY15 -

Ca^{++} had a denser cross-section than PE-BW40-GLY15. This finding clearly showed the beneficial effect of $CaCI_2$ cross-linking to achieved low WVP values.

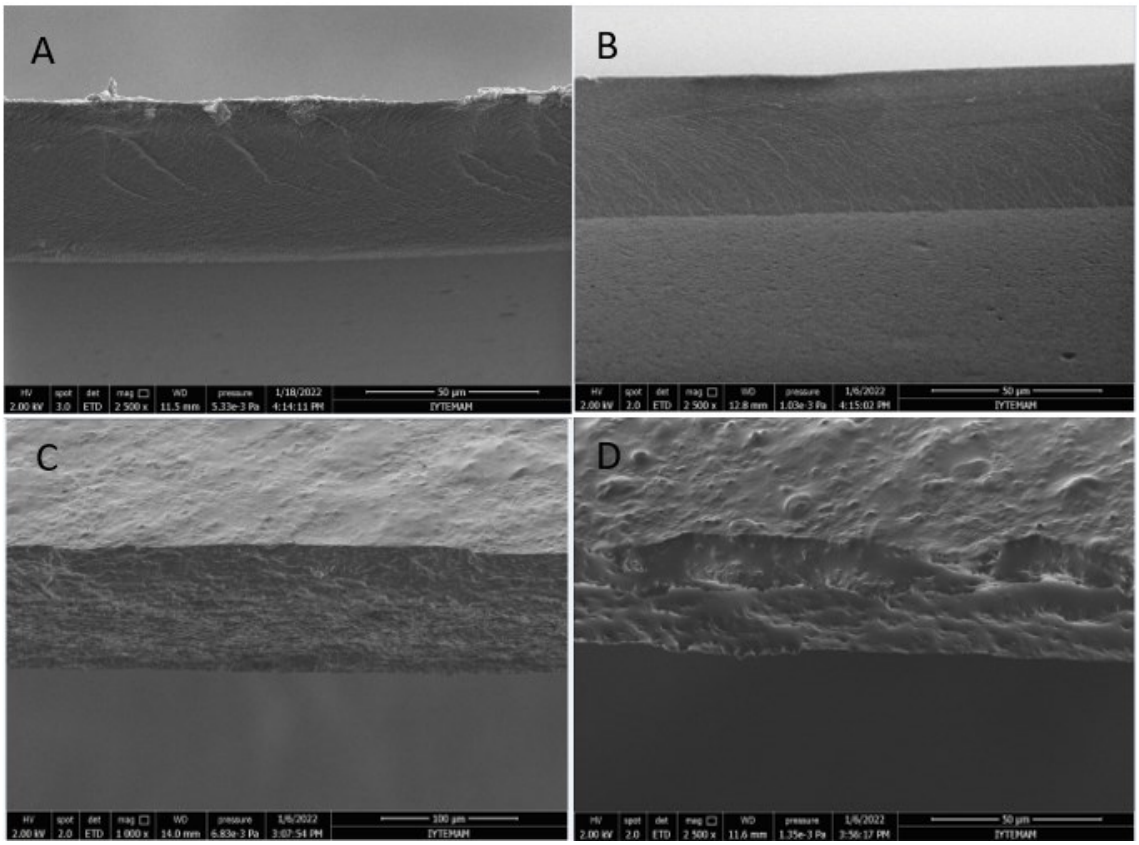

Figure 4.3. SEM cross section images of different films: (A) PE-GLY15 (B) PE-GLY15-Ca^{++} (C) PE-BW40-GLY15 (D) PE-BW40-GLY15 -Ca^{++}

The AFM surface images of pristine PE films (PE-GLY15, PE-GLY15-Ca^{++}) and selected PE-BW composite films, those performed well in WVP test (PE-BW40-GLY15, PE-BW40-GLY15 -Ca^{++}) are also seen in Fig. 4.4. The pristine PE films obtained with or without with $CaCI_2$ cross-linking contained too rough surfaces interrupted with sharp peaks, but no visible pores were observed at their surfaces (Fig 4.4A and 4.4B). In contrast, the PE-BW40-GLY15 had a less rough surface interrupted with several broadened peaks (Fig 4.4C) while PE-BW40-GLY15 -Ca^{++} films showed a smooth surface interrupted with several large craters having high and keen side walls (and 4.9D). However, the surfaces of both PE-BW composites also lacked deep pores. It seemed that the broadened peaks and large craters observed at composite surfaces are formed by melted embedded wax particles.

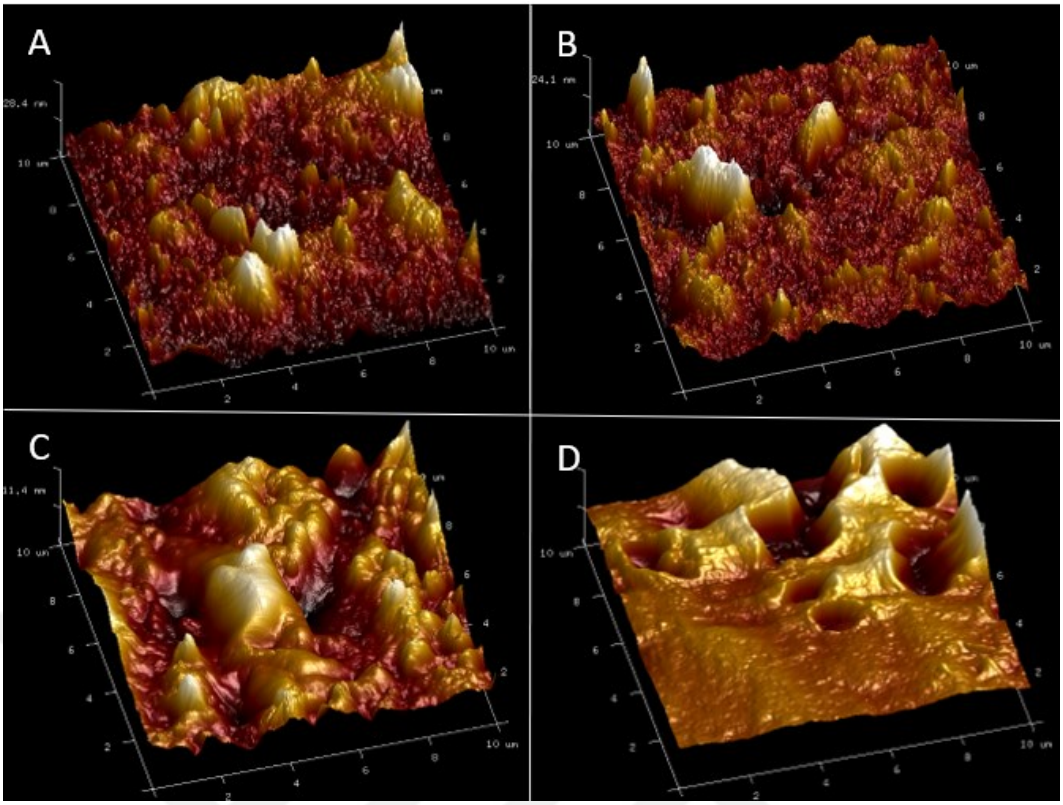

Figure 4.4: Topografic images of pectin-bees wax films: (A) PE-GLY15 (B) PE-GLY15-Ca^{++} (C) PE-BW40-GLY15 (D) PE-BW40-GLY15 -Ca^{++}

4.2. Development of moisture barrier pectin films by compositing with carnaubawax

4.2.1. Water vapour permeability of pectin-carnauba wax composite films

This part of the thesis involves use of carnauba wax (CW) in place of BW.

4.2.1.1. Effect of carnauba wax concentration on water vapour permeability of films

The studies with BW showed that the GLY concentration of 15% gave films with much lower WVP than GLY concentration of 30%. Thus, the GYL concentration was fixed at 15% for studies with CW. Moreover, the preliminary studies with CW showed that this

wax having higher melting point (m.p.= 82-86ºC) than BW (m.p.= 62-66ºC) cannot be used at the concentration of 40% (w/w of pectin). Thus, CW concentrations of 20 and 30% and GLY concentration of 15% were employed for the development of PE-CW composite films. Finally, composite films with CW became too brittle by $CaCI_2$ cross-linking, thus, they cannot be peeled from casting surface. Therefore, $CaCI_2$ cross-linking was also not applicable for PE-CW films. The results of WVP are presented in Figure 4.2. It is clear that CW is highly effective is reducing WVP of pectin-based composite films. The lowest WVP obtained with BW was 1.58 mm/m^2.day.kPa for PE-BW40-GLY15-Ca^{++} film. In contrast, PE-CW20-GLY15 and PE-CW30-GLY15 films gave WVP values of 0.34 and 0.16 mm/m^2.day.kPa that were almost 4.6 and 9.9-fold lower than that of PE-BW40-GLY15-Ca^{++} film. It is also important to note that these results meant that PE-CW30-GLY15 films also showed almost 50 and 24-fold lower WVP than pristine PE-GLY30 and PE-GLY15 films, respectively. Therefore, it was decided that the PE-CW films are more suitable for development of antimicrobial high moisture barrier films than PE-BW films.

4.2.1.2. Effect of carvacrol concentration on water vapour permeability of films

Carvacrol (CAR), a major antimicrobial essential oil component naturally found in oregano essential oil, was chosen as an antimicrobial to obtain antimicrobial high moisture barrier films. The effect of CAR incorporation on WVP of PE-CW films at 1, 2 and 3% concentrations are also given in Fig. 4.5. It is clear that the addition of CAR at 1 and 2% increased the WVP of PE-CW films significantly at a concentration dependant manner while composite films with CAR at 2 and 3% showed similar WVP values. Thus, is appeared that the maximum concentration of CAR should be 1%. It is important to note that PE-CW30-GLY15-CAR1% films shill showed better moisture barrier properties that pristine PE films and PE films incorporated with CAR AT 1%. The WVP of 1.47 mm/m^2.day.kPa determined for PE-CW30-GLY15-CAR1% film was 2.4-fold lower than WVPs of pristine PE-GLY15 film and PE-GLY15-CAR1% film, respectively. This result clearly proved that PE-CW30-GLY15-CAR1% is still a moisture barrier film superior than pristine or CAR incorporated PE films.

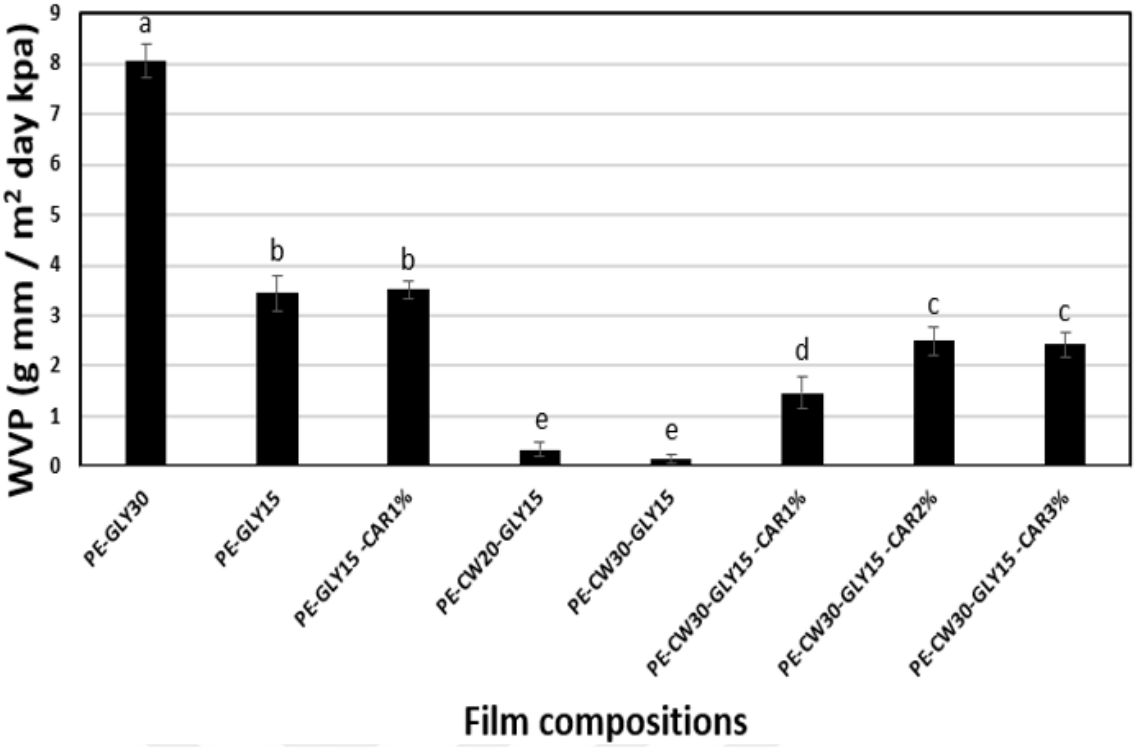

Figure 4.5. Effect of carnauba wax and carvacrol concentrations on water vapour permeabilities of different pectin-carnauba wax composite films (Different letters indicate significantly different values at $P < 0.05$).

4.2.2. Mechanical properties of pectin-carnauba wax composite films

The effects of incorporating CW and CAR into PE films are seen in Table 4.2. The TS values of PE-GLY15, PE-CW20-GLY15, and PE-CW30-GLY15 showed that addition of CW cuased limited changes in TS, EAB, and YM of films, respectively. However, incorporation of CAR alone or CW and CAR caused more remarkable changes in mechanical properties of films. It is important to note that the PE-GLY15-CAR1% showed the highest TS that indicate positive contribution of CAR on TS of pristine PE film. It seemed that the interaction of CAR with PE chains via H-bonding enhanced the mechanical strength of PE films. In contrast, incorporation of CAR into PE-CW films resulted with significant reductions in TS of films, indicating interruption of the network formed between CAR and PE by the extensive wax particles. In fact, the antimicrobial moisture barrier film developed (PE-CW30-GLY15-CAR1%) showed one of the lowest TS of PE-CW composites. In general, the EAB and YM of films varied within a narrow range, but it is important to note that the PE-CW30-GLY15-CAR3% film showed the lowest stiffness indicating loosening of the film-forming interacitons within the PE-CW

composites at the highest concentraiton of CAR. Thus, it is clear that the incorporation of CAR caused weakening of the mechanical strenths of PE-CW films.

Table 4.2 Effect of carnauba wax, glycerol and carvacrol concentrations on mechanical properties of different pectin-carnauba wax composite films

Film Composition	GLY (w/w)	CW (w/w)	CAR (w/v)	TS (MPa)	EAB(%)	YM (Mpa)
PE-GLY30	30%	-	-	10,86±1,14^{c}	4,56±2,13^{a}	4,79±0,90^{d}
PE-GLY15	15%	-	-	12,43±2,68^{b}	2,04±0,53^{c}	8,55±0,20^{a}
PE-GLY15-CAR1%	15%	-	1%	18,84±0,93^{a}	4,38±1,06^{a}	7,79±1,58^{a}
PE-CW30-GLY15	15%	30%	-	10,41±2,51^{c}	3,64±1,51^{ab}	5,66±0,69c
PE-CW20-GLY15	15%	20%	-	13,44±0,94^{b}	4,25±0,86^{a}	6,79±0,50^{b}
PE-CW30-GLY15-CAR1%	15%	30%	1%	5,16±0,56^{e}	1,35±0,17^{c}	5,50±0,59^{cd}
PE-CW30-GLY15-CAR2%	15%	30%	2%	8,71±1,39^{d}	2,51±0,36^{bc}	5,12±0,71^{cd}
PE-CW30-GLY15-CAR3%	15%	30%	3%	5,18±1,10^{e}	1,80±0,51^{c}	3,89±0,16^{e}

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

4.2.3. Antimicrobial Activity of pectin-carnauba wax composite films

Antimicrobial packaging plays a crucial role in inhibiting the growth of bacteria and fungi on food products. This active packaging method serves as a valuable alternative to traditional packaging for extending the shelf life of food products. Antimicrobial effect of CAR incorporated PE-CW composite films on *Listeria innocua and E.coli* are given in Table 4.3 and Figure 4.6. The CAR showed antimicrobial activity on both bacteria at a concentration dependant manner. The highest antimicrobial activity was observed 3% (w/v) CAR while the lowest antimicrobial effect was observed for PE-CW30-GLY15-CAR1%. PE-CW30-GLY15-CAR1% also showed slightly lower antimicrobial effect than PE-GLY15-CAR1%, suggesting slower release of CAR from PE-CW composite.

The zones determined for discs of CAR loaded PE-CW films against *L. innocua and E.coli* are also provided in Figure 4.7 and 4.8. It is worth to inform that although zones were identified against both bacteria they were sharper (zones were clearer) against *E.coli* than those against *L. innocua.*

Table 4.3. Antimicrobial effect of carvacrol incorporated pectin-carnauba wax composite films on *Listeria innocua and E.coli*

Film	Composition			Zone inhibition area (cm^2)	
	GLY (w/w)	CW (w/w)	CAR (w/v)	*L. innocua*	*E.coli*
PE-GLY15	15	-	-	1,2±0,1	0±0
PE-GLY15-CAR1%	15	-	1%	9,4±1,1	2,2±0,2
PE-CW30-GLY15	15%	30%	-	4,3±0,8	0±0
PE-CW30-GLY15-CAR1%	15%	30%	1%	6,2±0,5	1,5±0,4
PE-CW30-GLY15-CAR2%	15%	30%	2%	16±2,7	7,3±0,1
PE-CW30-GLY15-CAR3%	15%	30%	3%	19,4±1,1	10,5±2,2

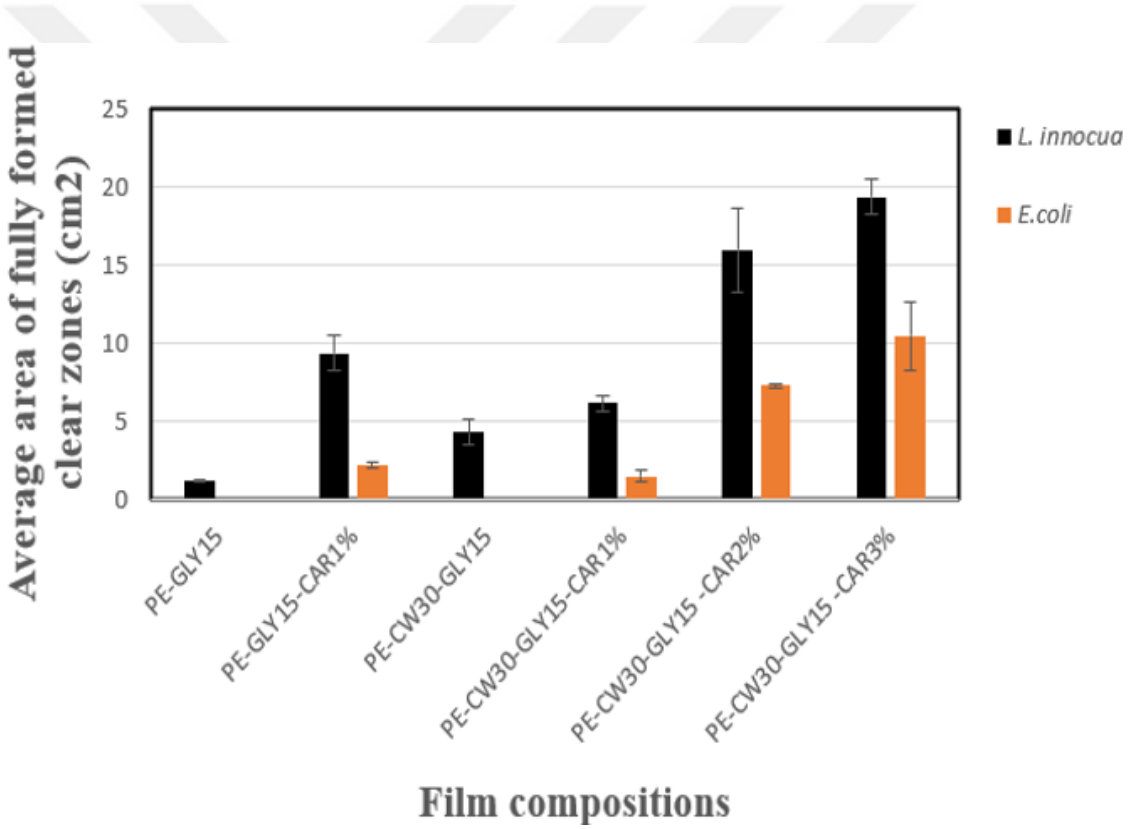

Figure:4.6: Antimicrobial effect of carvacrol incorporated pectin-carnauba wax composite films on *Listeria innocua* and *E.coli*

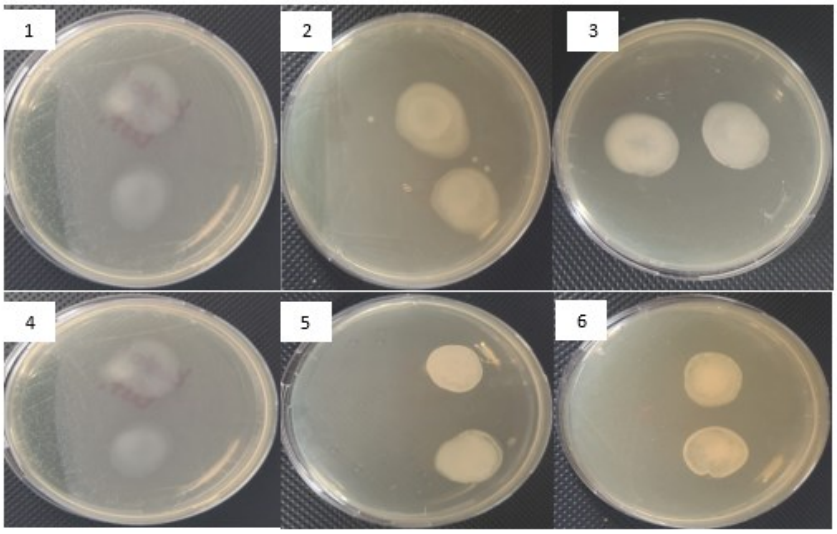

Figure 4.7: Antimicrobial effect of different films on *L. innocua* 1-PE-GLY15; 2-PE-CW30-GLY15; 3-PE-CW30-GLY15-CAR1%; 4-PE-CW30-GLY15-CAR%1; 5-PE-CW30-GLY15-CAR%2; 6-PE-CW30-GLY15-CAR3%

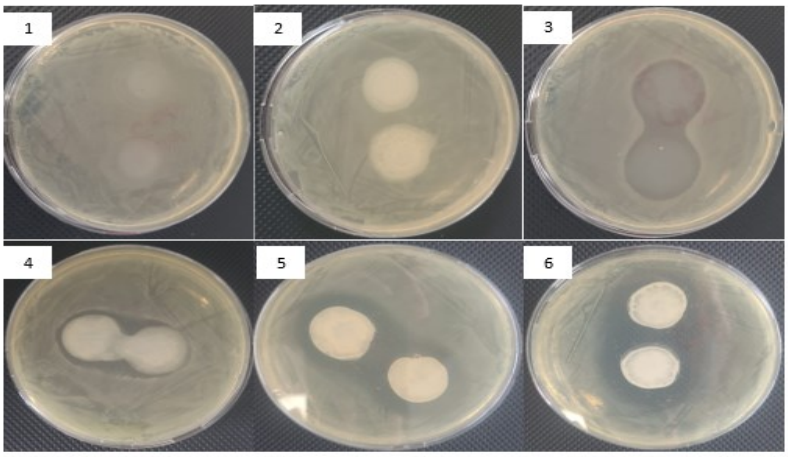

Figure 4.8: Antimicrobial effect of different films on *E.coli* 1-PE-GLY15; 2-PE-CW30-GLY15; 3-PE-GLY15-CAR1%; 4-PE-CW30-GLY15-CAR%1; 5-PE-CW30-GLY15-CAR%2; 6-PE-CW30-GLY15-CAR3%

4.2.4. Film thicknesses and transparencies of pectin-carnauba wax composite films

The thicknesses of PE and PE-CW composites are seen in Figure 4.9. In general, the PE-CW composite films were thicker than pristine PE films with the exception of PE-CW30-

GLY15-CAR%1. The highest film thickness was observed for PE-CW30-GLY15-CAR%2 and PE-CW30-GLY15-CAR%3 films. It seemed that CAR at high concentrations caused some major changes in films morphology. The incorporation of CW or CAR reduced the film transparency significantly. However, addition of both BW and CAR caused the highest reductions in film transparency.

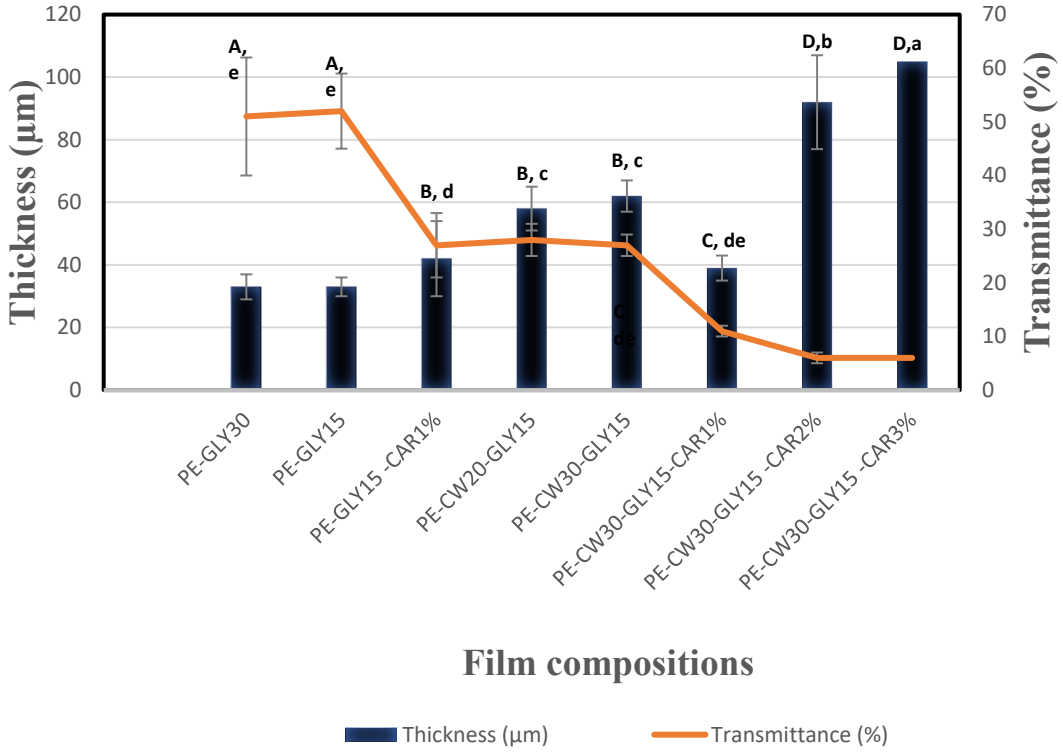

Figure 4.9: Effect of carnauba wax, glycerol and carvacrol concentrations on thickness and transparency of different pectin-carnauba wax composite films (Different letters indicate significantly different values at $P < 0.05$. The capital letters are for values of transmittance values. Lower case letters are for thickness values).

4.2.5. Morphology of pectin-carnauba wax composite films in SEM and AFM

The SEM cross-section images of PE and PE-CW composite films are seen in Figure 4.10. The PE-GLY15 films had a highly dense and pore free cross-section morphology (Fig. 4.10A). The PE-CW30-GLY15 films were also highly dense, but filled with extensive number of wax particles (Fig 4.10B). The incorporation of CAR increased the

porosity for both PE and PE-CW films (Fig 4.10 C, D, E, F). These results clearly showed why moisture barrier properties achieved by incorporation of CW was lost partially by incorporation of CAR.

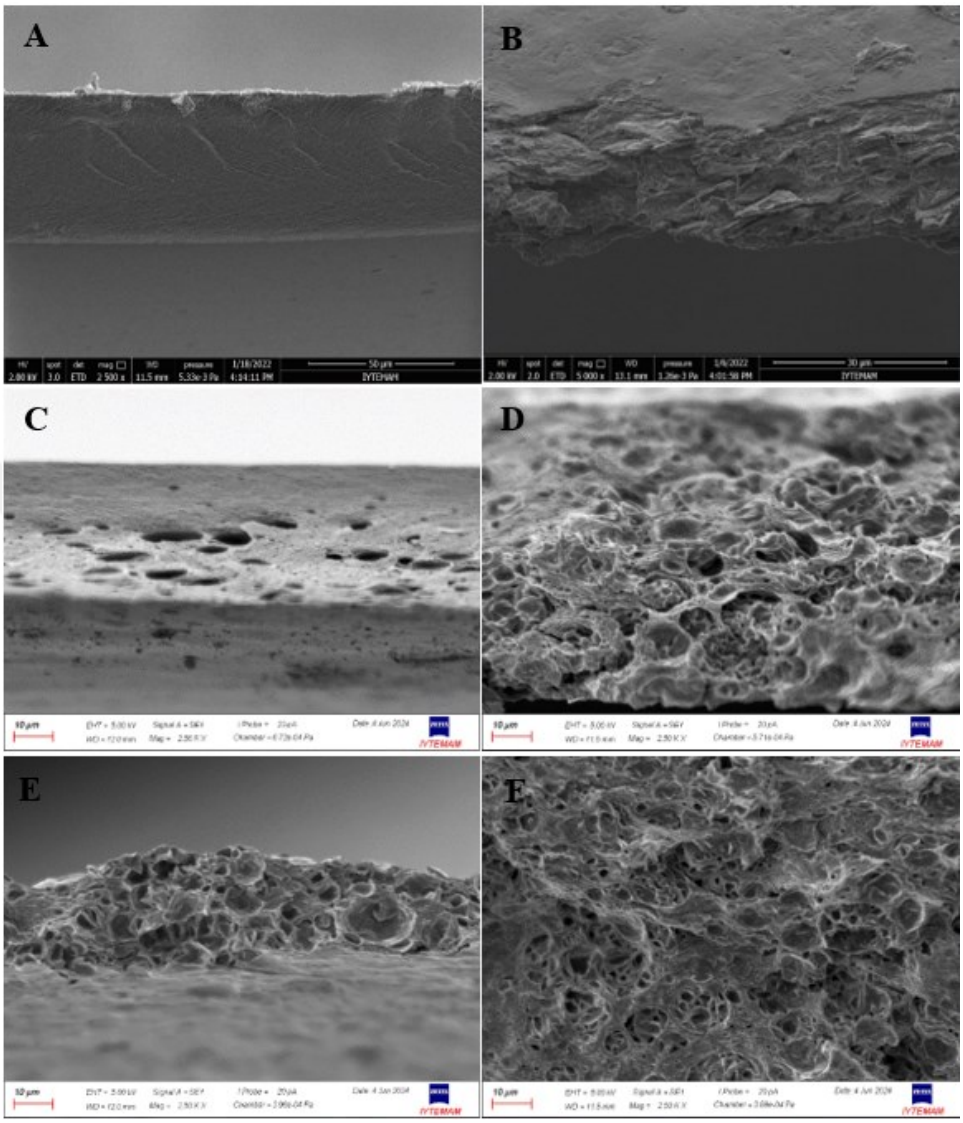

Figure 4.10: SEM cross section images of different films: (A) PE-GLY15; (B) PE-CW30-GLY15; (C) PE-GLY15-CAR1% (D) PE-CW30-GLY15 -CAR1% (E) PE-CW30-GLY15 -CAR2% (F) PE-CW30-GLY15 -CAR3%

The AFM of films are also seen in Figure 4.11. The pristine PE-GLY15 films showed a rough surface interrupted by sharp peaks (Fig. 4.11A) while PE-CW30-GLY15 films had a smoother surface interrupted by large valleys and broadened peaks. The incorporation of CAR at 1% caused apparent deep pores on the rough surfaces of pristine PE film while CAR at 1% caused deep craters and pores at the surface of PE-CW films. Similar observations were also observed in PE-CW composite films with 2 and 3% CAR. These

results confirmed SEM photos that also showed increased porosity of films by addition of CAR.

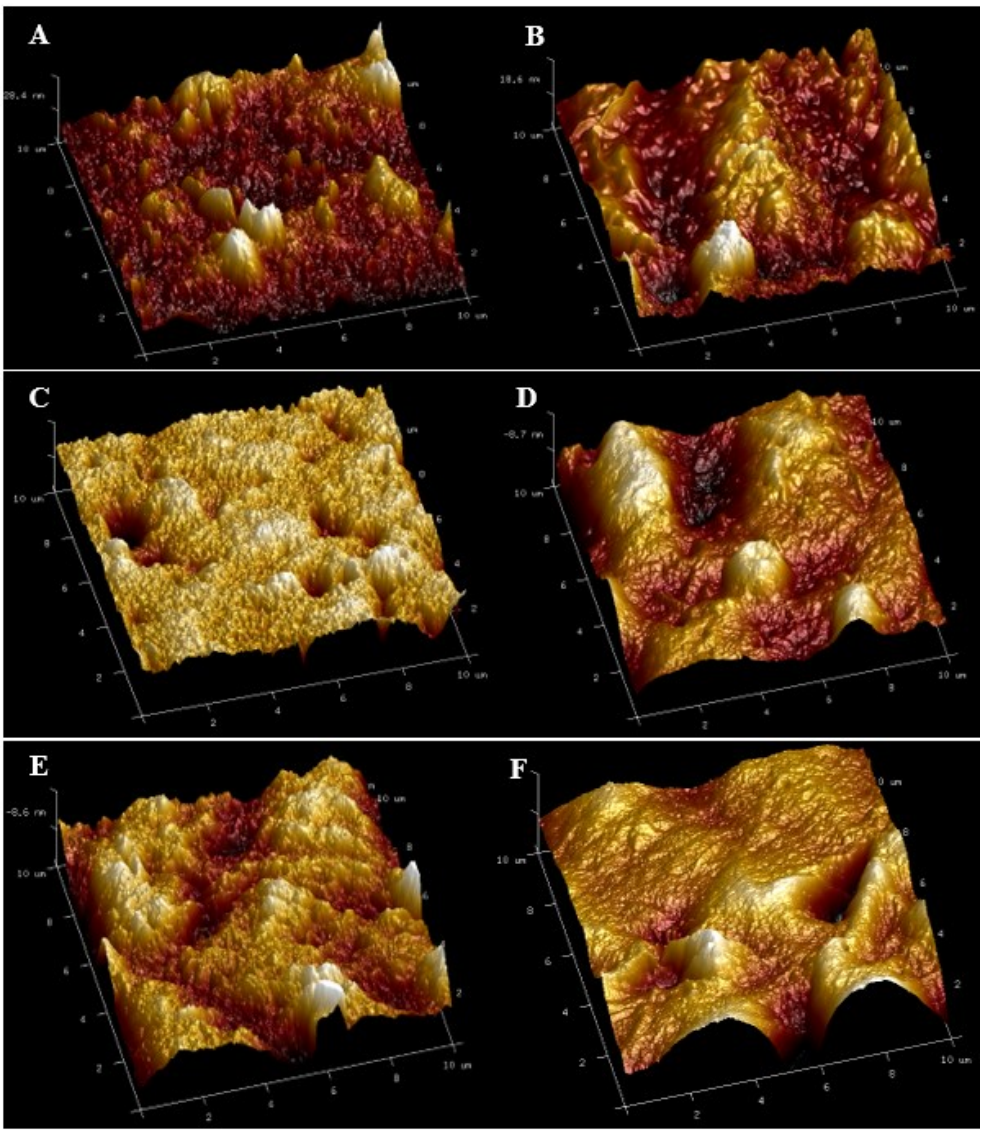

Figure 4.11: Topografic images of pectin-bees wax films: (A) PE-GLY15; B) PE-CW30-GLY15; (C)PE--GLY15-CAR1%; (D) PE-CW30-GLY15 -CAR1% (E) PE-CW30-GLY15 -CAR2% (F) PE-CW30-GLY15 -CAR3%

4.2.6. Solubility of Films

Prisdine PE films, PE-BW and PE-CW composite films all showed 100% solubility. This result clearly showed effective emulsification of both BW and CW within the PE film matrix. Thus, contact of films with water solubilizes films and suspended contents effectively.

CHAPTER 5

CONCLUSIONS

Composites of citrus pectin films with different waxes have been successfully obtained to develop moisture barrier packaging materials. Due to the high melting points of waxes the composites were formed by hot emulsification in the presence of an emulsifier under high-sheer homogenization.

Moisture barrier pectin-beeswax composite films were obtained only when films were cross-linked with $CaCl_2$ and wax content was increased up to 40%. Moisture barrier pectin-carnauba wax films were obtained by incorporating 30% wax into films. The $CaCl_2$ cross-linking and wax concentrations higher than 30% were not applicable to pectin-carnauba wax films as these practices gave highly brittle composites without film integrity. Pectin-carnauba wax composite films showed almost 50- and 10-fold better moisture barrier effect (lower WVP) than pristine pectin films and pectin-beeswax composite films, respectively. Therefore, carnauba wax was used for development of antimicrobial moisture barrier films.

Antimicrobial moisture barrier pectin-carnauba wax composite films effective on both gram-positive and gram-negative pathogenic bacteria have been developed by incorporation of essential oil carvacrol into films. The incorporation of carvacrol interfered with the moisture barrier properties of pectin-carnauba wax composite films, but the obtained antimicrobial films still showed 5.5-fold better moisture barrier properties than pristine pectin films. Moreover, they showed comparable moisture barrier properties with pristine pectin-beeswax composite films obtained by $CaCl_2$ cross-linking.

This thesis is one of the first example edible pectin film development studies targeting both moisture barrier properties and antimicrobial properties within the same film matrix.

Further studies are needed to screen more antimicrobials that show less interference with moisture barrier properties of developed composite pectin-carnauba wax composite films. The antimicrobial moisture barrier pectin films might find food applications as self-standing films or coatings for preservation of cheese blocks, sausages, intermediate moisture fruits and vegetables, and vegan food such as cheese and meat analogues.

Further studies are needed to develop innovative food applications with the developed films.

REFERENCES

Aitboulahsen, M.; El Galiou, O.; Laglaoui, A; Bakkali, M.;Hassani Zerrouk, M. Effect of Plasticizer Type and Essential Oils on Mechanical, Physicochemical, and Antimicrobial Characteristics of Gelatin, Starch, and Pectin-Based Films." *Journal of Food Processing and Preservation* **2020,** 44 (6). 10.1111/JFPP.14480 .

Anis, A.; Pal, K.,; Al-Zahrani, S. M. Essential oil-containing polysaccharide-based edible films and coatings for food security applications. *Polymers* **2021**, 13(4), 575. 10.3390/polym13040575.

American Society for Testing and Materials **2002a**. "Standard test method for tensile properties of thin plastic sheeting- D882-02".

American Society for Testing and Materials **2002b**. "Standard Test Method for Transparency of Plastic Sheeting-D 1746-97" .

Avramescu, S. M.; Butean, C.; Popa, C. V.; Ortan, A.; Moraru, I.; Temocico, G. Edible and Functionalized Films/Coatings Performances and Perspectives." *Coatings* **2020,** 10(7), 687. 10.3390/coatings10070687.

Bakkali, F., S. Averbeck, D. Averbeck, and M. Idaomar. Biological effects of essential oils–a review. *Food Chem. Toxicol.* **2008.** 46:446-475. 10.1016/j.fct.2007.09.106.

Benbettaïeb, N.; Debeaufort, F.;Karbowiak, T. Bioactive edible films for food applications: Mechanisms of antimicrobial and antioxidant activity". *Critical reviews in food science and nutrition* **2019**, 59(21), 3431 3455. 10.1080/10408398.2018.1494132.

Berenice, O.; Alvarez Pérez, Montanez, J. C; Aguilar, C. N; Molina R.R. Pectin-Candelilla Wax: An Alternative Mixture for Edible Films. *Journal of Microbiology, Biotechnology and Food Sciences* **2015,** *5*(2), 167. 10.15414/jmbfs.2015.5.2.167-171.

Bizymis, A. P.; Tzia, C. Edible Films and Coatings: Properties for the Selection of the Components, Evolution through Composites and Nanomaterials, and

Safety Issues. *Critical Reviews in Food Science and Nutrition* ***2022,*** 62 (31): 8777–92. 10.1080/10408398.2021.1934652.

Bosquez-Molina, E.; Guerrero-Legarreta, I.; Vernon-Carter, E.J. Moisture Barrier Properties and Morphology of Mesquite Gum-Candelilla Wax Based Edible Emulsion Coatings. *Food Research International* **2003**, 36(9-10), 885-893. 10.1016/S0963-9969(03)00097-8.

Bourtoom, T. Edible films and coatings: characteristics and properties. *International food research journal* **2008,** 15(3), 237-248. ISSN: 0127-7324

Caffall, K.H.; Mohnen D. The Structure, Function, and Biosynthesis of Plant Cell Wall Pectic Polysaccharides. *Carbohydrate research* **2009**, *344*(14), 1879-1900. 10.1016/j.carres.2009.05.021.

Cagri, A.; Ustunol Z.; Ryser E.T. Antimicrobial Edible Films and Coatings. *Journal of Food Protection* **2004**, 67 (4): 833–48. 10.4315/0362-028X-67.4.833.

Campos, C. A.; Gerschenson, L. N.; Flores, S. K. Development of Edible Films and Coatings with Antimicrobial Activity. *Food and bioprocess technology* **2011**, 4, 849-875. 10.1007/s11947-010-0434-1.

Çavdaroğlu, E.; Büyüktaş, D.; Farris, S.; Yemenicioğlu, A. Novel Edible Films of Pectins Extracted from Low-Grade Fruits and Stalk Wastes of Sun-Dried Figs: Effects of Pectin Composition and Molecular Properties on Film Characteristics. *Food Hydrocolloids* **2023,** 135 (February):108136. 10.1016/J.FOODHYD.2022.108136.

Chambi, H.; Grosso, C. Edible Films Produced with Gelatin and Casein Cross-Linked with Transglutaminase. *Food research international* **2006,** 39(4), 458-466. 10.1016/j.foodres.2005.09.009.

Cheng, J.; Wang, J.; Li, Z.; Chen, B.; Cui, L. Improving the mechanical and water-resistance properties of pea protein-based edible film via wet-heating Maillard reaction: Insights into the simultaneous effect of heating and Maillard reaction. *Food Packaging and Shelf Life* **2023,** *35*, 101024. 10.1016/j.fpsl.2023.101024.

Cheng, Y.; Zhai, X.; Wu, Y.; Li, C.;, Zhang, R.; Sun, C., Wang, W.; Hou, H. Effects of natural wax types on the physicochemical properties of starch/gelatin edible films fabricated by extrusion blowing. *Food chemistry* **2023,** 401, 134081. 10.1016/j.foodchem.2022.134081

Cortés-Rodríguez, M.; Villegas-Yépez, C.; González, J. H. G.; Rodríguez, P. E.; Ortega-Toro, R. Development and Evaluation of Edible Films Based on Cassava Starch, Whey Protein, and Bees Wax. *Heliyon* **2020,** 6(9). 10.1016/j.heliyon.2020.e04884.

de Oliveira Filho, J. G.; Bezerra, C. C. D. O. N;, Albiero, B. R.; Oldoni, F. C. A.; Miranda, M.; Egea, M. B.; Ferreira, M. D. New approach in the development of edible films: The use of carnauba wax micro-or nanoemulsions in arrowroot starch-based films. *Food Packaging and Shelf Life* **2020**, 26, 100589. 10.1016/j.fpsl.2020.100589.

Diyana, Z. N.; Jumaidin, R.; Selamat, M. Z.; Suan, M. S. M. Thermoplastic starch/beeswax blend: Characterization on thermal mechanical and moisture absorption properties. *International journal of biological macromolecules* **2021,** 190, 224-232. 10.1016/j.ijbiomac.2021.08.201.

Dubey, N. K.; Dubey, R. Edible films and coatings: An update on recent advances. *In Biopolymer-based formulations* **2020,** 675-695. 10.1016/B978-0-12-816897-4.00027-8

Eckholm, E. Mesopotamia: Cradle of haute cusine?. *The New York Times* **1985**.

Espitia, P. J. P.; Du, W. X.; de Jesús Avena-Bustillos, R.; Soares, N. D. F. F.; McHugh, T. H. Edible Films from Pectin: Physical-Mechanical and Antimicrobial Properties - A Review. *Food Hydrocolloids* **2014,** 35 (March):287–96. 10.1016/J.FOODHYD.2013.06.005.

Falguera, V.; Quintero, J. P.; Jiménez, A.; Muñoz, J. A.; Ibarz, A. Edible films and coatings: Structures, active functions and trends in their use. *Trends in Food Science & Technology* **2011,** *22*(6), 292-303. 10.1016/j.tifs.2011.02.004.

Gallo, J. A. Q.; Debeaufort, F.; Callegarin, F.; Voilley A. Lipid hydrophobicity, physical state and distribution effects on the properties of emulsion-based edible films. *Journal of Membrane Science* **2000,** 180(1), 37-46. 10.1016/S0376-7388(00)00531-7.

Galus, S.; Arik Kibar, E. A.; Gniewosz, M.; Kraśniewska, K. Novel Materials in the Preparation of Edible Films and Coatings—A Review. *Coatings* **2020,** Vol. 10, Page 674 10 (7): 674. 10.3390/coatings10070674.

Galus, S.; Gaouditz, M.; Kowalska, H.; Debeaufort, F. Effects of candelilla and carnauba wax incorporation on the functional properties of edible sodium caseinate films. *International Journal of Molecular Sciences* **2020,** 21(24), 9349. 10.3390/ijms21249349.

Ganiari, S.; Choulitoudi, E.; Oreopoulou, V. Edible and active films and coatings as carriers of natural antioxidants for lipid food. *Trends in Food Science & Technology* **2017,** 68, 70-82. 10.1016/j.tifs.2017.08.009.

Garavand, F.; Rouhi, M.; Razavi, S. H.; Cacciotti, I.; Mohammadi, R. Improving the Integrity of Natural Biopolymer Films Used in Food Packaging by Crosslinking Approach: A Review." *International Journal of Biological Macromolecules* **2017,** 104:687–707. 10.1016/j.ijbiomac.2017.06.093.

Gontard, N.; Thibault, R.; Cuq, B.; Guilbert, S. Influence of Relative Humidity and Film Composition on Oxygen and Carbon Dioxide Permeabilities of Edible Films. *Journal of Agricultural and Food Chemistry* **1996,** 44 (4): 1064–69. 10.1021/JF9504327.

Han, J. H. Edible films and coatings: a review. *Innovations in food packaging* **2014,** 213-255. 10.1016/B978-0-12-394601-0.00009-6.

Haq, M. A.; Hasnain, A.; Jafri, F. A.; Akbar, M. F.; Khan, A. Characterization of edible gum cordia film: Effects of beeswax. *LWT-Food Science and Technology* **2016,** 68, 674-680. 10.1016/j.lwt.2016.01.011.

Harholt, J.; Suttangkakul, A.; Vibe Scheller, H. Biosynthesis of Pectin. *Plant Physiology* **2010,** 153 (2): 384–95. 10.1104/PP.110.156588.

Hashemi, S. M. B.; Kaveh, S.; Abedi, E.; Phimolsiripol, Y. Polysaccharide-Based Edible Films/Coatings for the Preservation of Meat and Fish Products: Emphasis on Incorporation of Lipid-Based Nanosystems Loaded With Bioactive Compounds. *Foods* **2023,** 12(17), 3268. 10.3390/foods12173268.

Hassan, B.; Chatha, S. A. S.; Hussain, A. I.; Zia, K. M.; Akhtar, N. Recent Advances on Polysaccharides, Lipids and Protein Based Edible Films and

Coatings: A Review. *International Journal of Biological Macromolecules* **2018,** 109 (April):1095–1107. 10.1016/J.IJBIOMAC.2017.11.097.

Jeevahan, J.; Chandrasekaran, M. Nanoedible Films for Food Packaging: A Review. *Journal of Materials Science* **2019,** 54 (19): 12290–318. 10.1007/S10853-019-03742-Y .

Jovanović, J.;Ćirković, J.; Radojković, A.; Mutavdžić, D.; Tanasijević, G.; Joksimović, K.; Bakić, G.; Branković, G.; Branković Z. Chitosan and pectin-based films and coatings with active components for application in antimicrobial food packaging. *Progress in Organic Coatings* **2021,** 158, 106349. 10.1016/j.porgcoat.2021.106349.

Kocira, A.; Kozłowicz, K.; Panasiewicz, K.; Staniak, M.; Szpunar-Krok, E.; Hortyńska, P. Polysaccharides as Edible Films and Coatings: Characteristics and Influence on Fruit and Vegetable Quality—A Review. *Agronomy* **2021,** Vol. 11, Page 813 11 (5): 813. 10.3390/agronomy11050813.

Kumar, L.; Ramakanth, D.; Akhila, K.; Gaikwad, K. K. Edible films and coatings for food packaging applications: A review. *Environmental Chemistry Letters* **2022,** 1-26. 10.1007/s10311-021-01339-z.

Lara-Espinoza, C.; Carvajal-Millán, E.; Balandrán-Quintana, R.; López-Franco, Y.; Rascón-Chu, A. Pectin and pectin-based composite materials: Beyond food texture. *Molecules* **2018,** 23(4), 942. **10.3390/molecules23040942**

Lazaridou, A.; Biliaderis, C. G. Edible films and coatings with pectin. *Pectin: Technological and physiological properties* **2020,** 99-123. 10.1007/978-3-030-53421-9_6.

Le Normand, M.; Rietzler, B.; Vilaplana, F.; Ek, M. Macromolecular Model of the Pectic Polysaccharides Isolated from the Bark of Norway Spruce (Picea Abies). *Polymers* **2021**, Vol. 13, Page 1106 13 (7): 1106. 10.3390/POLYM13071106.

Li, J.; Wu, Y.; He, J.; Huang, Y. A new insight to the effect of calcium concentration on gelation process and physical properties of alginate films." *Journal of Materials Science* **2016,** 51, 5791-5801. 10.1007/s10853-016-9880-0

Lisitsyn A.; Semenova A.; Nasonova V.; Polishchuk .; Revutskaya N.;, Kozyrev I.; Kotenkova E. 2021. Approaches in Animal Proteins and Natural Polysaccharides Application for Food Packaging: Edible Film Production and Quality Estimation. *Polymers* **2020**, 13(10), 1592. 10.3390/polym.

Liu, J.; Bi, J.; McClements, D. J.; Liu, X., Yi, J.; Lyu, J.; Zhou, M.; Verkerk, R.; Dekker, M.; Wu, X.; Liu, D. Impacts of Thermal and Non-Thermal Processing on Structure and Functionality of Pectin in Fruit- and Vegetable-Based Products: A Review. *Carbohydrate Polymers* **2020,** 250 (December):116890. 10.1016/J.CARBPOL.2020.116890.

Maan, A. A.; Ahmed, Z. F. R; , Khan, M. K. I.; Riaz, A.; Nazir. A. Aloe Vera Gel, an Excellent Base Material for Edible Films and Coatings." *Trends in Food Science & Technology* **2021**, 116, 329-341. 10.1016/j.tifs.2021.07.035.

Milani, J. M.; Tirgarian, B. An overview of edible protein-based packaging: Main sources, advantages, drawbacks, recent progressions and food applications. *Journal of Packaging Technology and Research* **2020,** 4(1), 103-115. 10.1007/s41783-020-00086-w.

Milani, JM.; Nemati, A. Lipid-Based Edible Films and Coatings: A Review of Recent Advances and Applications. *Journal of Packaging Technology and Research,* **2022,** 6 (1): 11–22. 10.1007/s41783-021-00130-3.

Nastasi, J. R.; Kontogiorgos, V.; Daygon, V. D.; Fitzgerald, M. A. Pectin-Based Films and Coatings with Plant Extracts as Natural Preservatives: A Systematic Review. *Trends in Food Science & Technology* **2022,** 120, 193-211. 10.1016/j.tifs.2022.01.014.

Ngo, T. M. P.; Nguyen, T. H; Dang, T. M. Q.; Tran, T. X.; Rachtanapun, P. Characteristics and antimicrobial properties of active edible films based on pectin and nanochitosan. *International journal of molecular sciences*, **2020,** *21*(6), 2224. 10.3390/ijms21062224.

Nunes, C.; Silva, M.; Farinha, D.; Sales, H.; Pontes, R.; Nunes, J. Edible Coatings and Future Trends in Active Food Packaging–Fruits' and Traditional Sausages' Shelf Life Increasing. *Foods* **2023** , 12(17), 3308.. 10.3390/foods12173308.

Pedreiro, S.; Figueirinha, A.; Silva, A. S.; Ramos, F. Bioactive Edible Films and Coatings Based in Gums and Starch: Phenolic Enrichment and Foods Application. *Coatings* **2021,** 11:1393. 10.3390/coatings11111393.

Pérez, L. M.; Piccirilli, G. N.; Delorenzi, N. J.; Verdini, R. A. Effect of Different Combinations of Glycerol and/or Trehalose on Physical and Structural Properties of Whey Protein Concentrate-Based Edible Films. *Food Hydrocolloids* **2016,** 56, 352-359. 10.1016/j.foodhyd.2015.12.037.

Pérez-Vergara, L. D.; Cifuentes, M. T.; Franco, A. P.; Pérez-Cervera, C. E.; Andrade-Pizarro, R. D. Development and characterization of edible films based on native cassava starch, beeswax, and propolis. *NFS Journal* **2020,** 21, 39-49. 10.1016/j.nfs.2020.09.002.

Picot-Allain, M. C. N.; Ramasawmy, B.; Emmambux, M. N. Extraction, Characterisation, and Application of Pectin from Tropical and Sub-Tropical Fruits: A Review. *Food Reviews International* **2022,** 38 (3): 282–312. 10.1080/87559129.2020.1733008.

Prasad, J.; Dixit, A.; Sharma, S. P.; Mwakosya, A. W.; Petkoska, A. T.; Upadhyay, A.; Kumar, N.. Nanoemulsion-Based Active Packaging for Food Products. *Foods and Raw Material* **2024,** 12(1*).* 10.21603/2308-4057-2024-1-585.

Roman-Benn, A.; Contador, C. A.; Li, M. W.; Lam, H. M.; Ah-Hen, K.; Ulloa, P. E.; Ravanal, M. C. Pectin: An Overview of Sources, Extraction and Applications in Food Products, Biomedical, Pharmaceutical and Environmental Issues. *Food Chemistry Advances* **2023,** 2 (October):100192. 10.1016/J.FOCHA.2023.100192.

Ropartz, D.; Ralet, M. C. Pectin Structure. *Pectin: Technological and Physiological Properties* **2020,** October, 17–36. 10.1007/978-3-030-53421-9_2.

Sánchez-Ortega, I.; García-Almendárez, B. E.; Santos-López, E. M.; Amaro-Reyes, A.; Barboza-Corona, J. E.; Regalado, C. Antimicrobial Edible Films and Coatings for Meat and Meat Products Preservation. *The Scientific World Journal*, **2014**(1), 248935. 10.1155/2014/248935.

Sothornvit, R.; Krochta, J. M. Plasticizers in edible films and coatings. In *Innovations in food packaging* **2005,** pp. 403-433. Academic Press. 10.1016/B978-012311632-1/50055-3.

Suhag, R.; Kumar, N., Petkoska, A. T.; Upadhyay, A. Film formation and deposition methods of edible coating on food products: A review. *Food Research International* **2020,** 136, 109582. 10.1016/j.foodres.2020.109582.

Summo, C.; De Angelis, D. The Importance of Edible Films and Coatings for Sustainable Food Development. *Foods* **2022,** 11(20), 3221. 10.3390/foods.

Szulc, J.; Machnowski, W.; Kowalska, S.; Jachowicz, A.; Ruman, T.; Steglińska, A.; Gutarowska, B. Beeswax-modified textiles: method of preparation and assessment of antimicrobial properties. *Polymers* **2020,** 12(2), 344. 10.3390/polym12020344.

Tavassoli-Kafrani, E.; Shekarchizadeh, H.; Masoudpour-Behabadi, M. Development of Edible Films and Coatings from Alginates and Carrageenans." *Carbohydrate Polymers* **2016,** 137:360–74. 10.1016/j.carbpol.2015.10.074.

Umaraw, P.; Verma, A. K. Comprehensive review on application of edible film on meat and meat products: An eco-friendly approach. *Critical reviews in food science and nutrition* **2017,** *57*(6), 1270-1279. 10.1080/10408398.2014.986563.

Ünalan, İ. U.; Arcan, I.; Korel, F.; Yemenicioğlu, A. Application of active zein-based films with controlled release properties to control Listeria monocytogenes growth and lipid oxidation in fresh Kashar cheese." *Innovative Food Science & Emerging Technologies* **2013,** *20*, 208-214. 10.1016/j.ifset.2013.08.004.

Vieira, T. M.; Moldão-Martins, M.; Alves, V. D. Design of Chitosan and Alginate Emulsion-Based Formulations for the Production of Monolayer Crosslinked Edible Films and Coatings. *Foods* **2021,** Vol. 10, Page 1654 10 (7): 1654. 10.3390/FOODS10071654.

Wu, Y.; Wu, H.; Hu, L. Recent Advances of Proteins, Polysaccharides and Lipids-Based Edible Films/Coatings for Food Packaging Applications: A Review." *Food Biophysics* **2024,** 19 (1): 29–45. 10.1007/S11483-023-09794-7.

Yao, S.; Wang, B. J.; Weng, Y. M. Preparation and characterization of mung bean starch edible films using citric acid as cross-linking agent. *Food Packaging and Shelf Life* **2022,** 32, 100845. 10.1016/j.fpsl.2022.100845.

Yemenicioğlu, A. Edible food packaging with natural hydrocolloids and active agents. *CRC Press* **2022**. 10.1201/9780429329890.

Yıldırım-Yalçın, M,; Sadıkoğlu, H.; Şeker, M. Characterization of Edible Film Based on Grape Juice and Cross-Linked Maize Starch and Its Effects on the Storage Quality of Chicken Breast Fillets. *Lwt* **2021,** 142, 111012. 10.1016/j.lwt.2021.111012.

Yuan, D.; Hao, X.,; Liu, G.; Yue, Y.; Duan, J. A Novel Composite Edible Film Fabricated by Incorporating W/O/W Emulsion into a Chitosan Film to Improve the Protection of Fresh Fish Meat. *Food Chemistry* **2022,** 385, 132647. 10.1016/j.foodchem.2022.132647.

Zhang, L.; Chen, D.; Yu, D.; Regenstein, J. M.; Jiang, Q.; Dong, J.; Chen, W.; Xia, W. Modulating Physicochemical, Antimicrobial and Release Properties of Chitosan/Zein Bilayer Films with Curcumin/Nisin-Loaded Pectin Nanoparticles. *Food Hydrocolloids* **2022,** 133, 107955. 10.1016/j.foodhyd.2022.107955.

Zhang, Y.; Kong, Q.; Niu, B.; Liu, R.; Chen, H.; Xiao, S.; Wu, W.; Zhang, W.; Gao, H. The Dual Function of Calcium Ion in Fruit Edible Coating: Regulating Polymer Internal Crosslinking State and Improving Fruit Postharvest Quality. *Food Chemistry* **2024,** 138952. 10.1016/j.foodchem.2024.138952.

Zhang, Y.; Simpson, B.K.; Dumont. M.J. Effect of Beeswax and Carnauba Wax Addition on Properties of Gelatin Films: A Comparative Study. *Food bioscience* **2018,** 26, 88-95. 10.1016/j.fbio.2018.09.011 .

APPENDICES

APPENDIX A. The Data Tables of The Experimental Findings

Table A1. Effect of beeswax and glycerol concentrations and $CaCI_2$ induced cross-linking on water vapour permeabilities of different pectin-beeswax composite films

Film	Glycerol (w/w)	Bees Wax (w/w)	CaCl2 (3%, w/v)	WVP (g mm /m² day kPa)
PE-GLY30	30%	-	-	8,06±0,33[a]
PE-GLY15	15%	-	-	3,45±0,36[c]
PE-BW30-GLY30	30%	30%	-	4,60±1,1[b]
PE-BW30-GLY30 -Ca++	30%	30%	+	4,53±0,04[b]
PE-BW30-GLY15	15%	30%	-	4,45±0,35[b]
PE-BW30-GLY15 -Ca++	15%	30%	+	4,89±0,14[b]
PE-BW40-GLY30	30%	40%	-	4,28±0,1[b]
PE-BW40-GLY30 -Ca++	30%	40%	+	1,93±0,05[d]
PE-BW40-GLY15	15%	40%	-	3,08±0,23[c]
PE-BW40-GLY15 -Ca++	15%	40%	+	1,58±0,46[d]

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

Table A2. Effect of carnauba wax, glycerol and carvacrol concentrations on water vapour permeabilities of different pectin-carnauba wax composite films

Film	Glycerol (w/w)	Carnauba wax (w/w)	CAR (w/v)	WVP (g mm / m2 day kPa)
PE-GLY30	30%	-	-	8,06±0,33[a]
PE-GLY15	15%	-	-	3,45±0,36[b]
PE-GLY15 -CAR1%	15%	-	1%	3,52±0,18[b]
PE-CW20-GLY15	15%	20%	-	0,34±0,15[e]
PE-CW30-GLY15	15%	30%	-	0,16±0,09[e]
PE-CW30-GLY15 -CAR1%	15%	30%	1%	1,47±0,31[d]
PE-CW30-GLY15 -CAR2%	15%	30%	2%	2,50±0,29[c]
PE-CW30-GLY15 -CAR3%	15%	30%	3%	2,43±0,24[c]

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

Table A3. Effect of beeswax and glycerol concentrations and $CaCl_2$ induced cross-linking on mechanical properties of different pectin-bees wax composite films

Film Composition	Glycerol (w/w)	Beeswax (w/w)	CaCl2 (%3, w/v)	TS (MPa)	EAB(%)	YM (Mpa)
PE-GLY30	30%	-	-	10,86± 1,14^{bcd}	4,56± 2,13^{bc}	4,79± 0,90^{c}
PE-GLY15	15%	-	-	12,43± 2,68^{ab}	2,04± 0,53^{ef}	8,55± 0,20^{ab}
PE-BW30-GLY30	30%	30%	-	9,10± 2,53^{de}	4,48± 1,28^{bc}	3,84± 1,41^{cd}
PE-BW30-GLY30-Ca++	30%	30%	+	13,97± 3,81^{a}	6,81± 2,29^{a}	4,27± 0,95^{cd}
PE-BW30-GLY15	15%	30%	-	7,83± 1,63^{ef}	5,22± 0,79^{b}	3,05± 0,79^{d}
PE-BW30-GLY15 -Ca++	15%	30%	+	5,83± 3,77^{f}	1,18± 0,41^{f}	7,25± 1,92^{b}
PE-BW40-GLY30	30%	40%	-	7,17± 1,90^{ef}	2,38± 0,81^{def}	3,17± 1,30^{d}
PE-BW40-GLY30 -Ca++	30%	40%	+	9,40± 3,44^{cde}	3,05± 2,42^{cde}	4,50± 0,97^{cd}
PE-BW40-GLY15	15%	40%	-	12,27± 2,55^{abc}	1,63± 0,44^{ef}	9,03± 1,86^{a}
PE-BW40-GLY15 -Ca++	15%	40%	+	14,60± 2,31^{a}	3,71± 1,02^{cd}	6,77± 0,59^{b}

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

Table A4. Effect of carnauba wax, glycerol and carvacrol concentraitons on mechanicalproperties of different pectin-carnauba wax composite films

Film Composition	Glycerol (w/w)	Carnauba wax (w/w)	CAR (w/v)	TS (MPa)	EAB(%)	YM (Mpa)
PE-GLY30	30%	-	-	10,86± 1,14^{c}	4,56± 2,13^{a}	4,79± 0,90^{d}
PE-GLY15	15%	-	-	12,43± 2,68^{b}	2,04± 0,53^{c}	8,55± 0,20^{a}
PE-GLY15 - CAR1%	15%	-	1%	18,84± 0,93^{a}	4,38± 1,06^{a}	7,79± 1,58^{a}
PE-CW30-GLY15	15%	30%	-	10,41± 2,51c	3,64± 1,51ab	5,66± 0,69c
PE-CW20-GLY15	15%	20%	-	13,44± 0,94b	4,25± 0,86a	6,79± 0,50b
PE-CW30-GLY15 -CAR1%	15%	30%	1%	5,16± 0,56e	1,35± 0,17c	5,50± 0,59cd
PE-CW30-GLY15 -CAR2%	15%	30%	2%	8,71± 1,39d	2,51± 0,36bc	5,12± 0,71cd
PE-CW30-GLY15 -CAR3%	15%	30%	3%	5,18± 1,10e	1,80± 0,51c	3,89± 0,16e

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

Table A5. Effect of carnauba wax, glycerol and carvacrol concentrations on thickness and transparency of different pectin-carnauba wax composite films

Film Composition	Glycerol (w/w)	Carnauba wax (w/w)	CAR (w/v)	Thickness (µm)	Transmittance (%)
PE-GLY30	30%	-	-	33±5^{e}	51±7^{a}
PE-GLY15	15%	-	-	33±4^{e}	52±11^{a}
PE-GLY15 -CAR1%	15%	-	1%	42±3^{d}	27±7^{b}
PE-CW20-GLY15	15%	20%	-	58±12^{c}	28±6^{b}
PE-CW30-GLY15	15%	30%	-	62±7^{c}	27±3^{b}
PE-CW30-GLY15-CAR1%	15%	30%	1%	39±5de	11±2^{c}
PE-CW30-GLY15 -CAR2%	15%	30%	2%	92±4^{b}	6±1^{d}
PE-CW30-GLY15 -CAR3%	15%	30%	3%	105±15^{a}	6±1^{d}

*Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).

Table 3. Effect of beeswax and glycerol concentrations and CaCl2 induced cross-linking on thickness and transparency of different pectin-beeswax composite films

Film Composition	**Glycerol (w/w)**	**Bees wax (w/w)**	**$CaCl_2$ (3%, w/v)**	**Thickness (µm)**	**Transmittance (%)**
PE-GLY30	30%	-	-	33 ± 5^{f}	51 ± 7^{a}
PE-GLY15	15%	-	-	33 ± 4^{f}	52 ± 11^{a}
PE-BW30-GLY30	30%	30%	-	73 ± 5^{b}	15 ± 1^{d}
PE-BW30-GLY30 - Ca++	30%	30%	+	61 ± 4^{de}	16 ± 1^{cd}
PE-BW30-GLY15	15%	30%	-	81 ± 6^{a}	24 ± 6^{b}
PE-BW30-GLY15 - Ca++	15%	30%	+	68 ± 6^{c}	23 ± 3^{b}
PE-BW40-GLY30	30%	40%	-	61 ± 4^{e}	20 ± 3^{bc}
PE-BW40-GLY30 - Ca++	30%	40%	+	66 ± 9^{c}	20 ± 3^{bc}
PE-BW40-GLY15	15%	40%	-	66 ± 4^{cde}	18 ± 2^{cd}
PE-BW40-GLY15 - Ca++	15%	40%	+	66 ± 4^{c}	15 ± 2^{d}

**Values are given as mean ± standard deviation. Values shown in each row indicated by different letters are significantly different ($P < 0.05$).