Characterization of PLA-PHB Based Active Packaging Film, and Its Application on Oysters

by

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Abstract

Biodegradable material polylactic acids (PLA) and polyhydroxybutyrate (PHB) were designed as the matrices for active food packaging applied on oysters. In this study, the performance of bioplastics was first evaluated through the comparison of PLA-PHB and Ethanol vinyl alcohol (EVOH, as reference group). Detailedly, mechanical properties such as tensile strength, elongation at break, oxygen transmission rate (OTR), water vapor permeation (WVP); active properties including releasing, antibacterial and antioxidant were determined. Then the effectiveness of preserving oysters was valued via the contrast of PLA-PHB and PLA-PHB with fennel oil (PLA-PHB-FEN). More specifically, bacteria, pH value, TVB-N, texture profile and free amino acids of oyster samples were tested. The results showed that the overall properties of PLA-PHB are nearly comparable to those of EVOH except for the oxygen barrier property. The release of fennel oil in 65% ethanol ranked highest and the maximum concentration is 38.82 μg/mL. Though the release in water is very low, PLA-PHB-FEN still presented almost 1 log inhibition on selected bacteria. When applying on oysters, the count number of aerobic and anaerobic bacteria were both 1 log CFU/g lower in PLA-PHB-FEN than in PLA-PHB and EVOH. In addition, the pH value of oyster samples was 5.98 and 5.96 respectively in PLA-PHB and EVOH on Day 12th when the total aerobic count reached the acceptable limit, while it was 5.79 on Day 16th in PLA-PHB-FEN packaging. The dominant free amino acids in oyster samples are glutamic acid, histidine, alanine and arginine, accounting for about 92.91% of total amount of amino acids. Oyster samples have less total amount of amino acids in PLA-PHB-FEN

than other two packaging, indicating that there were less bacteria activity due to the inhibitory action of fennel. We concluded that this biodegradable packaging is effective to extend the shelf-life of oyster samples for 2 to 3 days with the function of fennel oil.

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Chapter 1 Introduction

Oyster is one of the most popular seafoods worldwide. To achieve the best taste and prevent the loss of nutrition, oysters are usually consumed raw, therefore, how to minimize the number and inhibit the growth of microorganism and pathogens are of great importance. Irradiation (Jakabi et al., 2003) and high-pressure hydraulic treatment (Cruz-Romero et al., 2008) are two preservation methods commonly used for one-time sterilize to reduce the initial number of microbes.

Despite the strong antimicrobial effects, damage on texture, leading to the leakage of tissue liquid and denaturation of proteins, could be well utilized by microorganisms, subsequently cause the spoil of body. Therefore, some mild preservative technologies are taken into consideration such as application of additives, antimicrobial packaging, etc.

It is also noteworthy that fresh shucked oysters are usually stored in jars for the market serving, however, jar is space consuming during transportation, and can hardly be used in a vacuum way, where soft packaging can take over the role. Traditional food packaging functions as a barrier segregating external force and some essential elements for microorganisms such as moisture, oxygen or even heat and light to provide a fundamental protection. And currently, it derives out many multifunctional packaging such as active and intelligent packaging. Among them, antimicrobial packaging is the one of the most popular active packaging.

Literally, antimicrobial packaging is composed of antimicrobial agents and packaging materials. Antimicrobial agents can be categorized into four groups based on the origin: chemicals from synthesis, extracts from animal organs, fermentation products from microorganisms and essential oils from plants (Juneja et al., 2012). Regardless of inhibitory

effect, natural agent would be the first choice due to consumers' psychology regarding food. Fennel oil, known for its antispasmodic, diuretic, anti-inflammatory, analgesic effects, is commonly used in pharmaceutical and cosmetic products. In addition, it is also a popular flavoring agent in food products (Senatore et al., 2013). However, the study of fennel oil used as antimicrobial material (active element) in packaging film is rarely reported. In fact, the constituents of fennel such as trans-anethole, eugenol, limonene and estragole are all with the ability to inhibit bacterial growth (Kubo et al., 2008 ; Senatore et al., 2013). The working mechanisms could be concluded to a couple of following ways: 1. To break through the cell membrane and then damage the membrane integrity, leading to the loss of electrolytes as well as proteins and sugars(Diao et al., 2014); and 2. To impact on the respiration of cell via inhibiting the nicotinamide-adenine dinucleotide (NADH) oxidase (Kubo et al., 2008).

As to the active element, there are several ways to add antimicrobial agents into food packaging such as directly apply the agents onto packaging materials with or without chemical bonds, or using pads as a media indirectly contacting with products(Appendini & Hotchkiss, 2002). However, no matter via direct or indirect way, the chance for agents to contact with food is significantly enhanced, resulting in several problems: 1. The hazard of food safety would be increased; 2. The release/ effectiveness of active element would soon be completed and cannot reach a long-term goal; and 3. The contacting area is restricted that the quality of food cannot be integrally controlled. Therefore, the advantages show up when blending antimicrobial active element with polymers directly while extrusion show up. Polylactic acids (PLA) and polyhydroxy butyrate (PHB) are two popular bioplastics used as base matrices in antimicrobial packaging, taking advantage of their biodegradability, good processability and moldability. Nevertheless, the difficulty throughout the researches on biodegradable material is pointed to

their mechanical properties because of their incompact structure. According to Zhang's and other studies, the mechanical properties of PLA-PHB blend was improved to the maximum extent when the portion of PLA and PHB is 3:1 since the PHB particles finely dispersed in PLA crystal space and act as nucleating agent to enhance the recrystallization(Zhang & Thomas, 2011).

Besides raw materials of packaging, the selection of manufacturing techniques of packaging film is also very important. The manufacture of packaging film is usually categorized into two types: blown vs. casting. Comparing to blown, casting process provides a higher gage uniformity, rate of production, as well as higher transparency, and thermoforming performance, due to the generation of smaller crystals and lower crystallinity with a faster cooling process (TedBrink, https://www.slideshare.net/TedBrink/cast-versus-blown-film).

In order to develop a biodegradable packaging film to prolong the shelf-life of oysters, we first characterize and evaluate the mechanical properties including tensile strength, elongation at break, WVP, OTR, releasing activity, antibactericidal and antioxidant ability the of PLA-PHB, PLA-PHB-FEN, and EVOH films. Then, apply films to packing oysters to verify the effectiveness of the active packaging on extending the shelf-life of oysters. The measurement index comprises pH value, TVB-N, aerobic and anaerobic bacteria count, texture profile analysis and free amino acids determination.

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Chapter 2 Literature review

Oysters

Oysters, more precisely, edible oysters are commonly consumed by human for their high contents of protein and amino acids but low in fat. They are also rich in zinc, iron, calcium, selenium, vitamin A and vitamin B12. Typical types of edible oysters are Belon oyster, eastern oyster, Olympia oyster, Pacific oyster, and the Sydney rock oyster.

The eastern oyster, *Crassostrea virginica*, (phylum Mollusca, class Bivalvia, order Ostreoid, family Ostreidae), currently distributed in a great diversity of habitats along the western Atlantic Ocean from the Canadian Maritime Provinces to the Gulf of Mexico, Panama and the Caribbean Islands (Jenkins et al., 1997). Like all oysters, eastern oysters is a bivalve mollusk with a hard calcium-carbonaceous shell, its valves are asymmetrical with the left valve generally thicker and more deeply cupped than the right (Yonge 1960; Galtsoff 1964 cited by National Marine Fisheries Service, 2007) (National Marine Fisheries Service, 2007). Eastern oysters settle on the left valve leaving the right valve always on top.

Oyster plays a very important role in most estuaries along the Atlantic and Gulf coasts, contributing to the integrity and functionality of estuarine ecosystems. They consume and remove nitrogen-containing compounds (nitrates and ammonia), phosphates, plankton, detritus, bacteria, and dissolved organic matter from the water. As reported, their water filtration ability could be as high as 10 L h^{-1} g^{-1}dry tissue weight (Newell & Langdon, 1996). Because of their high clearance rate, they are evaluated as a possible bioremediation tool to reduce contaminant

loading in marsh-estuarine systems (Breitburg et al. 2000 cited by National Marine Fisheries Service, 2007).

Every coin has two sides, when they filter water, bacteria, heavy metal, toxin are also accumulated and concentrated in their body. The first identified lethal disease is caused by *Perkinsus marinus* (1950 by John Mackin), followed by *Minchinia nelson* (1966 by Haskin, Stauber and Mackin) (National Marine Fisheries Service, 2007). *Vibrio* bacteria is reported to be the foremost pathogen in molluscan shellfish causing foodborne diseases as they are naturally occurring in marine and estuarine environments (Hlady, 1997). *Vibrios* including *Vibrio vulnificus* (*V. vulnificus*) and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) are estimated to cause approximately 5,000 foodborne infections annually in the United States (Mead et al., 1999), and the incidence of *Vibrio* infections has shown a sustained increase since 2000 (Control & Prevention, 2010).

Raw consumption of oysters is highly preferred. Therefore, oysters are sold as whole in shell or as shucked meat in the markets. Half shucked oysters are only traded in the restaurants or the retail stores where they are shucked in order to meet the customers' needs of freshness. At the same time, shucked oysters are required to be raw during the shucking process so that they can be consumed as raw meats at restaurants or home level (Wheaton, 2007).

To reduce the microorganism in raw oysters as much as possible, several technologies are profoundly studied.

(Quevedo et al., 2005) tried to use ice immersion as a postharvest treatment method to reduce the number of *V. vulnificus* in oysters. Unwashed whole oysters were immersed rapidly in ice for 3 hrs. and then were refrigerated at 45 °F (7.2 °C). They found that the numbers of *V. vulnificus* generally declined in treated samples compared with controls, however the total

heterotrophic bacteria and fecal coliforms increased, therefore, they did not support the use of ice immersion as a postharvest method.

High hydraulic pressure (HHP) is the most popular way to reduce bacteria in oysters. (Calik et al., 2002) treated *V. parahaemolyticus* in broth cultures and inoculated live Pacific oysters (*Crassostrea gigas*) at pressure 241, 276, 310, and 345 MPa, they found that at 345 MPa for 30 or 90 s was the optimum condition to reduce *V. parahaemolyticus* to the level that below present FDA action levels. (Kural & Chen, 2008) did a series of trials to find a best combination of HHP and temperature for a 5-log reduction of *V. vulnificus* in oysters, it turned out that to achieve a > 5-log reduction in the counts of V. vulnificus, pressure treatment needs to be conducted at pressure levels of ⩾ 250 MPa at − 2 or 1 °C. (Cruz-Romero et al., 2008) raised the pressure to 500 MPa and 800 MPa, after pressure treatment, the oysters were stored at 2 °C. They found that oysters treated under 500 or 800 MPa for 5 min were thoroughly free from *Vibrio spp.*, but there were *Pseudomonas spp.* detected. Cruz-Romero et al. also did some researches on microbiological and physicochemical change of oysters during chill storage after HPP (Cruz-Romero et al., 2008), and comparison of the effect of HPP, cool pasteurization, and traditional pasteurization treatments on oysters (Cruz-Romero et al., 2007). Other researchers (Collins et al., 2005 ; Lingham et al., 2016) also give their trust on HP treatment in the preservation of oysters. However, when (Prapaiwong et al., 2009) treated their oyster samples with HP treatment at 250 to 400 MPa for 1 to 3 min, they found that HP treatment is effective in reducing microbial loads in raw oysters, however, those bacteria who survived the treatment proliferate to an unexpected high number, even higher than untreated samples during refrigeration, and they are more likely to cause spoilage and alter the organoleptic properties of treated oysters.

Apart from HPP, irradiation is another popular technology used to preserve oysters. (Mahmoud, 2009) treated pure culture and inoculated oysters with 0.0, 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, and 3.0 kGy X-ray at 22 °C and 50–60% relative humidity, they found that greater than a 6-log reduction of *V. vulnificus* was achieved with 0.75, 1.0, 3.0 kGy X-ray in pure culture, half shell and whole shell oysters, respectively. And treatment with 0.75 kGy X-ray could reduce the microorganisms in half shell oysters to less than the detectable limit (< 1 log CFU/g). (Mahmoud & Burrage, 2009) used X-ray treatment on reducing *V. parahaemolyticus* as well. (Jakabi et al., 2003) exposed oyster samples to gamma radiation (^{60}Co), finding that a dose of 3.0 kGy can be considered effective in reducing *Salmonella* and *V. parahaemolyticus* by 5 to 6 $\log_{10}$ units without changing odor, flavor and appearance.

Other methods such as combination of ozone water and chitosan (Rong et al., 2010), warm pasteurization with supercritical fluid CO_2 as an agent (Meujo et al., 2010) have been proved to have an effective effect on preservation of oysters.

In spite of the high efficiency of these sterilization techniques, accompanied problems cannot be ignored. As mentioned, the bacteria survived from HPP could grow at an unexpected high speed even than untreated one, this phenomenon could be explained by the over fluid extruded from body containing abundant nutrition and essential elements for microorganism, providing a better environment for them to breed. Irradiation is intolerable to microorganism, but whether it will change physiology of foods is still unknown, which could be another potential hazard to consumers. And since oysters are more preferable to be consumed in raw, consumers would not psychologically accept the use of additives.

Antimicrobial packaging is a relatively new subject in food area. It could provide a mild, non-destructive and long period preservation to food contents.

Food packaging

Food packaging has become an indispensable part to food products as it not only provides basic protection to foods such as isolating external oxygen, moisture, heat and light, which could lead to microbial and oxidative spoilage, but also facilitates distribution during processing and supply chain (Ahvenainen, 2003). In addition, it plays a role as conveying information to customers about what the product is, what the compositions are, when it is produced, how to keep and so on.

With the increasing attention to food quality and safety, people start to focus on development of packaging, therefore, active packaging and intelligent packaging are being designed for the purpose of 'changing the condition of the packed food to extend shelf-life or to improve safety or sensory properties, while maintaining the quality of the packaged food' and ' monitoring the condition of packaged foods to give information about the quality of the packaged food during transport and storage' (definition of active and intelligent packaging released by ACTIPAK-FAIR CT98-4170 (Ahvenainen, 2003)).

Antimicrobial packaging

To minimize processing procedures as well as maintaining safety and quality of food, antimicrobial packaging, one of the active packaging concepts, has been studied over the past decades. As the active substance, antimicrobial agents are added into food packaging via several techniques such as placing sachets/pads containing volatile antimicrobial agents into packages, coating antimicrobials onto polymer surface, immobilizing antimicrobials to polymer by ionic or covalent bonds, and blending antimicrobial agents with polymers directly while extrusion (Appendini & Hotchkiss, 2002). We purpose to study the characterizations and application of antimicrobial packaging through blending as it has significant advantages of less additive

contacting with foods and the controlled release of antimicrobial compounds. These antimicrobials will be released to food content or the headspace of package in a relative long period so that they can inhibit or retard the growth of microorganism more effectively.

Packaging materials

Packaging material can be classified into two categories according to the degradability: non-degradable and degradable. To reduce the irreversible pollution caused by non-degradable materials, degradable materials have become preferable in packaging field. Despite of their degradability, they usually cannot meet the requirements of good mechanical properties as non-degradable one, therefore, a couple of ways to strengthen their structure are studied such as adding plasticizers, making into multi-layers and so on. In our research, we aimed to make both degradable and non-degradable packaging with the same manufacturing method and compare their characterizations to see if our degradable materials could take the place of non-degradable one.

1. Non-degradable material: Ethylene vinyl alcohol (EVOH)

EVOH is the polymerization products of ethanol and vinyl alcohol followed by hydrolysis. It is well known for its excellent mechanical properties, especially its high oxygen barrier performance. EVOH has the lowest oxygen permeability among these commonly used packaging materials, except for polyvinylidene chloride (PVDC). The oxygen permeability of EVOH (EVAL® resin with 27 mol% ethylene) is about 0.006 cc.mil/100 in^2·day·atm at 23℃ and 0% RH (Data from EVAL Americas (Houston, TX)), which is over 4 orders of magnitude lower than that of polypropylene, polyethylene, and polystyrene (Mokwena & Tang, 2012). Taking advantages of its high oxygen barrier performance, EVOH materials have been recently developed as matrices for active packaging to prevent the food content and active substances

from oxygen deterioration(Muriel-Galet et al., 2012). On the other hand, EVOH is water sensitive, which means when exposed to high relative humidity environment, its barrier characteristics diminishes significantly. Based on this property, EVOH can be molded in the form of multilayer structure where it can be protected by moisture resistance films (Mokwena & Tang, 2012 ; López-Rubio et al., 2005).

2. Degradable materials: Polylactic acid (PLA) and Polyhydroxybutyrate (PHB)

PLA enjoys the world second highest consumption volume of bioplastic materials. It derives from bioderived monomers, which can be produced to a great extent by the microbial fermentation of agricultural by-products, especially the carbohydrate-rich substances. Another reason that PLA films aroused such great concern is due to the degradability. Large polymer backbone is cut off via hydrolysis in the first step when exposed to moisture, and then naturally metabolized by microorganisms (Auras et al., 2004).

Properties of PLA depend on the composition of isomers, polymerization methods and temperature control during the process. In general, PLA has comparable mechanic properties to those of polyethylene terephthalate (PET) and better than these of polystyrene (PS). Given that stiffness and brittleness caused by low crystallinity, considerable methods have been applied for PLA modification to extend the usability of PLA in food packaging industry, such as the addition of modifiers, nanotechnology, copolymerization and blending (Arrieta et al., 2014c). Recently it was found that PLA, as films, is also a good matrix for active substances, the releasing rate of actives substances could be retarded from weeks to months with the packed of PLA.

PHB is either a kind of popular bioplastic. It is one of the best-known classes of Polyhydroxyalkanoates (PHA) and generated as energy storage molecule when microorganisms are lack of energy sources during metabolism, which has been proved to take place in the

presence of a variety of bacteria (Bucci et al., 2005). Due to its biodegradability, biocompatibility, and its manufacture from renewable resources, PHB is now of great interest to polymer industry. However, one of the weaknesses is the relatively high crystallinity and brittleness. Thus, plasticizers as modifiers or blending with other degradable polymer are the two ways to achieve higher flexibility and elongation at break (Freier et al., 2002).

2.1. Blends of PLA and PHB

PHB has similar melting point to PLA allowing physical blending both polymers in the same melting state. Zhang et al. studied the properties of blends of PLA and PHB with different ratios. They found that the mechanical properties are significantly improved when the portion of PLA and PHB is 3:1 since the PHB particles finely dispersed in PLA crystal space and act as nucleating agent to enhance the recrystallization (Zhang & Thomas, 2011).

Plasticizer

Even though PHB can improve the mechanical properties of PLA, inherent brittleness of both PLA and PHB still cannot be avoided, therefore, plasticizers are supplemented in most occasions. Plasticizers work by embedding themselves to polymer chain and thus lower the glass transition temperature, which enables the polymers to be more flexible and processible. Arrieta et al. studied the function of the D-limonene as plasticizer and the structural and surfaces properties, as well as functional and mechanical properties of PLA-PHB blended film. The results indicated a general improvement in those properties (Arrieta et al., 2014a). Moreover, oligomeric lactic acid (OLA), poly (ethylene glycol) (PEG) and acetyl-tri-n-butyl citrate (ATBC) have also been proved to be useful plasticizers (Arrieta et al., 2014b ; Arrieta et al., 2014c ; Armentano et al., 2015).

The new type of epoxy functionalized chain extender TMP-6000 has moderate epoxy groups in its structure. It can be widely used in PLA, PHA and other bio-based polyester products, functioning as binder that combine broken molecules by heating, therefore improve the mechanical properties and the processing performance.

N, N'-Ethylenebis (EBS) is a synthetic wax used as a dispersing agent in plastic applications to facilitate and stabilize the dispersion of solid materials to enhance processability.

Essential oil

Essential oils (EOs) are aromatic substances generally obtained from plant via distillation, expression, solvent extraction and so forth. The greatest use of EOs is in food (as flavorings), perfumes and pharmaceuticals (for their functional properties). Another important usage is as antimicrobial substances. The foremost mechanism of action is their hydrophobicity, which enables them to separate the bacterial cell membrane and mitochondria, leading to structure destruction and higher permeation (Burt, 2004).

Well known for its antispasmodic, diuretic, anti-inflammatory, analgesic effects, fennel oil are commonly used as a constituent of pharmaceutical and cosmetic products (Senatore et al., 2013). It is also a popular flavoring agent in food products (Charles et al., 1993). Shahat compared three fennel cultivars on their antimicrobial activity, concluding that most essential oil samples presented higher antibacterial activity against gram positive than gram negative bacteria (Shahat et al., 2011). Many chemical components of extracted fennel oil were reported to have the antimicrobial effect such as *trans*-anethole, eugenol, limonene and estragole (Kubo et al., 2008 ; Senatore et al., 2013).

Application of antimicrobial food packaging

There are many researches on characterizing or modifying antimicrobial food packaging. (Ramos et al., 2012 ; Cerisuelo et al., 2012 ; Persico et al., 2009) studied the characterization of carvacrol in polypropylene, EVOH, and nanocomposite. (Becerril et al., 2007) studied the antimicrobial activity of cinnamon contained active packaging against *E. coli* and *S. aureus*. tried to improve active chitosan film with rosemary essential oil.

However, the study of real application on food products is rare. (Balaguer et al., 2013) applied gliadin films incorporating with cinnamaldehyde on bread and cheese spread foodstuffs, they found that gliadin film with 3% cinnamaldehyde is highly effective against fungal growth. Mold growth was observed on sliced bread after 27 days of storage at 23 °C with active packaging, while fungal growth appeared on control bread around the fourth day. In the cheese spread, no fungi were observed after 26 days of storage at 4 °C when the product was packaged with the active film and fungi start to grow on control cheese after 16 days of storage. (Duan et al., 2007) made chitosan based packaging film with 60% lysozyme for the preservation of Mozzarella cheese, the results showed that the active film could completely inhibit the growth of mold and reduce yeast population as well. (Chen et al., 2014) employed chitosan as packaging matrix, incorporating with citral β-Cyclodextrin inclusion complex, to prolong the shelf-life of fresh beef, the results indicated that the shelf-life of beef samples could be extended for about 5 days comparing with control samples. It has also been proved that EVOH film blended with cinnamaldehyde could prolong the shelf-life of snakehead for 3-4 days (Ma et al., 2017).

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Chapter 3: Comparison of three films: ethylene vinyl alcohol (EVOH), polylactic acid-polyhydroxybutyrate (PLA-PHB) and polylactic acid-polyhydroxybutyrate-Fennel oil blends

Abstract

The characterization of three films, ethylene vinyl alcohol (EVOH), polylactic acid-polyhydroxybutyrate (PLA-PHB) and polylactic acid-polyhydroxybutyrate-Fennel oil blends (PLA-PHB-FEN) were profiled and compared through the mechanical properties tests and active properties tests. The results showed that mechanical properties of these films are almost comparable to each other except elongation at break and oxygen transmission rate (OTR). The elongation at break value of EVOH is 212.70±49.41% while that of PLA-PHB with or without fennel oil is around 5%, which means EVOH has a 40-fold greater endurance to strain than PLA-PHB. The OTR value of EVOH is 6.51 $cm^3/m^2 \cdot d \cdot 0.1MPa$ when PLA-PHB has a 513.9 $cm^3/m^2 \cdot d \cdot 0.1MPa$ transmission rate, however, it is noticeable that the addition of fennel oil has significantly improved the OTR, that is 253.25 $cm^3/m^2 \cdot d \cdot 0.1MPa$. Active properties tests were also conducted including releasing, antioxidant and bactericidal tests, indicating that PLA-PHB-FEN film is more applicable on oily foods, and fennel oil contributed to an 8% antioxidant ability and one log antibacterial ability comparing to PLA-PHB film.

Introduction

Since 1983, the world demand of polymer has grown from 45 million tons to 250 million tons. AMI's analysis looks in detail at where and how polymers are used worldwide, disclosing a fact people may not being aware of that packaging is the one who has come to dominate the end-use markets for polymer (AMI), and food packaging is approximately 50% (by weight) of total packaging sales (IFT). Polymers have supplied most of common packaging materials because of their desired features like softness, lightness and transparency. However, the ecological problem caused by the non-degradability cannot be ignored.

Environmental concerns are intensifying the interest in agricultural and forestry resources, or by-products and wastes as alternative feedstocks for packaging. Polymers derived from renewable resources could be classified into three groups based on their production methods (Petersen et al., 1999 ; Weber et al., 2002):

1. Polymers directly extracted/removed from natural materials (mainly plants). For example, polysaccharides such as starch and cellulose and proteins like casein and wheat gluten.

2. Polymers produced by classical chemical synthesis from renewable bio-derived monomers. A good example is polylactic acid (PLA), a biopolyester polymerized from lactic acid monomers. The monomer itself is produced via fermentation of carbohydrate feedstock.

3. Polymers produced by microorganisms or genetically transformed bacteria. The best-known biopolymer types are the Polyhydroxyalkanoates (PHA), mainly polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). The family of PHA functions in microorganisms as energy substrates and for carbon storage.

The benefits of using biobased packaging are no doubt considerable, nevertheless, the problems accompanying are threefold: performance, processing and cost. Reasons are low molecular weight, loose structure, sensitive to water/moisture and so on, which are also the

reasons for why biodegradable materials could be consumed or digested by nature. Therefore, no single biobased material will satisfy all potential markets or applications (Weber et al., 2002).

PLA and PHB are widely accepted as potential substitutions for synthetic polymers. PLA has an overall good performance compared with standard thermoplastics. Auras et al. compared PLA and polyethylene terephthalate (PET) and oriented polystyrene (OPS) on their physical and mechanical performance (Auras et al., 2006), the results showed that PLA has a good oxygen barrier performance that is perfect for breathable products, which makes it become one of the most commercialized biodegradable polymer, other than this, PLA has a higher tensile strength and similar chemical resistance. Another important point is that PLA is very biodegradable under certain conditions and can be processed using standard industrial machinery (Petersen et al., 1999). PLA and PHB are between two extremes where synthetic polymers are highly resistant to hydrolysis while nature materials (such as cellulose and starch) are extremely hydrophilic (Scott & Wiles, 2001). Several authors have reported that PLA and PHB blends at a ratio of 3:1 shows a greatly improved properties (Abdelwahab et al., 2012 ; Arrieta et al., 2014a ; Arrieta et al., 2014b ; Zhang & Thomas, 2011).

Even though their characterizations have been modified with the existence of each other, it cannot be denied that they are still away from synthetic ones. Thus, researchers try to add functional elements such as essential oils to take the advantages of their active nature.

Fennel oil is commonly used as a constituent of pharmaceutical and cosmetic products because of its antispasmodic, diuretic, anti-inflammatory, analgesic effects (Senatore et al., 2013). It is also a popular flavoring agent in food products (Charles et al., 1993). Like most kinds of essential oil added as antimicrobials, one of the mechanisms of action is their hydrophobicity, which enables them to separate the bacterial cell membrane and mitochondria, leading to

structure destruction and higher permeation (Burt, 2004). Shahat compared three fennel cultivars on their antimicrobial activity, concluding that most essential oil samples presented higher antibacterial activity against gram positive than gram negative bacteria (Shahat et al., 2011).

The aim of this research is to develop a biodegradable packaging film and comparable the properties to those of non-biodegradable one to see the potential of PLA-PHB blends to replace non-biodegradable material.

Materials and methods

Materials

Raw materials for food packaging such as EVOH (E105B, containing 44 mol% of ethylene), PLA (2003D) and P(3,4HB) were all purchased from Kuraray Technology Co., Ltd. (Shanghai, China). Fennel oil as the antimicrobial active element was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Film casting

PLA and PHB pellets were first dried to remove the water content because of their hydrophilicity. 75% PLA and 25% (w/w) PHB pellets were blended with 1% TMP 6000 chain extender, 0.2% Irganox245 antioxidant and 1% N, N'-Ethylenebis stearamide (EBS) lubricant using double screw extruder (LSSJ-20, Kechuang Rubber Plastic Mechanical Equipment Co., Ltd., Shanghai, China), then the melt polymer was extruded as a strand going through cold water bath. The new granules were added with 6% (w/w) FEN oil, melted and blended by single screw extruder followed by a casting machine. The heating temperature ranged from 135℃ to 170℃, and the screw rotation rate was 60 rpm while the roller rotation rate was 6.0 rpm.

EVOH neat film was also prepared as reference group. The heating temperature ranged from 16℃ to 210℃, and the screw rotation rate was 45 rpm while the roller rotation rate was 7.5 rpm.

Three formulations were obtained and named as EVOH, PLA-PHB and PLA-PHB-FEN.

Physical and mechanical properties

1. Tensile properties tests

Tensile properties tests including tensile strength and elongation at break were conducted under ambient temperature by an Instron universal testing machine (Model 1130) according to (ASTM, 2010). Prior to testing, films were cut into 1.5 × 15 cm strips perpendicularly to the direction of flow, and the thickness of each strip was measured using the digital micrometer (Mitutoyo, Osaka, Japan). The testing conditions used were: cross head speed of 50 mm/min and load cell of 0.1 kN. Each film was tested at least 3 times and the reported values were the average of 3 close measurements.

2. Oxygen transmission rate (OTR)

OTR was carried out under the condition of 23°C and 65% RH with a OXTRAN model 2/21 ML (Mocon, Minneapolis, United States), following the instruction of (ASTM, 1982). A thickness measurement was carried out followed by a 4 to 10 hours' testing.

3. Water vapor permeation (WVP)

Water vapor transmission rate (WVTR) was tested using a PERMATRAN-W, model 1/50G (Mocon, Minneapolis, United States), following the instruction of (ASTM, 2003). The test was performed under the condition of 37.8 °C, 100% permeant RH. The WVP of the film was calculated as the following equation:

$$WVP = WVTR \times N/\Delta P \quad (1)$$

where WVTR is measured by instrument ($g/m^2 \cdot d$), N is the thickness of the film (m) and ΔP is the vapor pressure across the film (2×10^5 Pa).

4. Optical property tests

The transmittance and haze were tested by a Light Transmittance Rate and Haze Tester, model WGT/S (Precision Instrument Company, Shanghai, China) according to (ASTM, 1997).

Active properties test

1. Releasing test

Releasing test was conducted following the procedures described by Ma, et al. (Ma et al., 2017). Four aqueous food simulants were employed to imitate the release of active element in food, they are water, 3% acetic acid solution (v/v), 10% ethanol solution (v/v) and 65% ethanol solution (v/v) corresponding to aqueous, acidic, alcoholic and fatty foods respectively. 2.00g films were double-sided, totally immersed in 1 L simulants. The containers were stored in a 4°C fridge in where oyster samples were placed. 1 mL of each simulant were sampled periodically and then the concentration of FEN oil in simulants was measured with the gas chromatography-mass spectrometric (GC-MS) analysis.

GC-MS analysis was performed on an Agilent 6890N combined with Agilent 5975B inert MSD, equipped with a Rxi®-5SiL MS column (30 m×0.25 mm, 0.25 μm film thickness). Oven temperature was set at 60 °C for 3 min, then to 250 °C at 10°C/min, carrier gas was helium at 1.5 ml/min; injection volume was 1.0 μl.

2. Antioxidant activity

The antioxidant activity of films was determined using the stable free radical diphenylpicrylhydrazyl (DPPH) method according to Yichao (Ma et al., 2017). The 12.8 mg DPPH (Bean Town Chemical, NH, United States) was dissolved with 250 mL absolute ethanol to make 0.1 mol/L DPPH solution. Three pieces of 12 cm^2 of each film were soaked in 50 mL 95% ethanol solution, and placed in 65 °C water bath for 3 hours to let the active element release. Another 50% ethanol solution was also prepared as the blank group. Cross-mixed DPPH solution, sample solution and ethanol solution and let them stand for 30 mins at room temperature covered with aluminum foil. The absorbance of mixtures was determined at 517 nm with a UV spectrometer (Helios omega UV/VIS spectrophotometers, Thermo Scientific, United States). The free radical scavenging rate was expressed by the following equation:

$$\text{Scavenging rate (\%)} = [1- (Abs_{sample}-Abs_{blank})/Abs_{DPPH}]\times 100\% \quad (2)$$

where Abs_{sample} is the absorbance of the mixture of DPPH solution and sample solution; Abs_{blank} is the absorbance of the mixture of sample solution and 50% ethanol solution; Abs_{DPPH} is the absorbance of the mixture of DPPH solution and 50% ethanol solution. Each sample was assayed three times.

3. Bactericidal activity

E. coli O157:H7 and *Staphylococcus aureus* as the representative gram negative and gram positive bacteria (Cho et al., 2005 ; Eaton et al., 2008) were selected to fulfil the antibacterial activity test. The method is described in (Ma et al., 2017) with a little modification. Films weighing 0.5g each were sterilized under ultraviolet light for 30 mins, then added into tubes with 10 mL tryptic soy broth (TSB). These tubes were stored at 4°C for 5 days to allow active element release. Cells of *E. coli O157:H7* and *Staphylococcus aureus* from cultured agar plates was transferred to TSB with shaking at 37 °C for 12 hours to allow their growth to come to a

stationary phase, then the two bacteria solutions were diluted to around 10^7 CFU/mL. Diluted bacteria solution (100 μL) was inoculated into film sample tubes to achieve an initial level of 10^5 CFU/mL. These culture media were incubated in a shaking incubator at room temperature for another 12 hours. Antibacterial activity was then measured by counting the colonies in appropriate dilutions of each sample tube. All tests were performed in triplicate.

Statistical analysis

Prior to one-way and two-way ANOVA testing, means and standard deviations were calculated. One-way ANOVA was used for the significance differences among mechanical properties and active properties of three films, accompanied with the Tukey's HSD (honest significant difference) test at 95% confidence level ($P<0.05$). The R software (version 3.3.3) was used to perform these analyses.

Results and Discussion

Mechanical properties

Table 3. 1 Mechanical properties of films. All values except WVP and OTR are means ± standard deviations from measurements (n=3).

Category	Thickness (mm)	Tensile strength (MPa)	Elongation at break (%)	WVP (g·m/[m2·s·Pa])	OTR (cm3/m2·d·0.1MPa)	Haze (%)	Transmittance (%)
EVOH	0.044±0.000	30.38±3.54^a	212.70±49.41^b	1.57E-09	6.51	4.01±1.18^a	86.48±1.05^c
PLA-PHB	0.044±0.002	41.20±2.04^b	4.93±0.75^a	7.05E-09	513.9	11.31±0.60^c	78.26±1.77^a
PLA-PHB-FENNEL	0.053±0.001	41.49±2.00^b	6.90±2.70^a	6.94E-09	253.25	7.65±1.51^b	81.58±0.50^b

* Superscript a, b and c represents significant difference among three films.

The evaluated value of thickness, tensile strength, elongation at break as well as WVP, OTR, haze and transmittance were listed in Table 3.1 EVOH shows an extremely bigger elastic

deformation than PLA-PHB, that the value of elongation at break is about 40-fold greater, at the same time, the difference of tensile strength among three films is also apparent based on the P value ($P<0.05$). Comparing to the remarkable difference between EVOH and PLA-PHB, FEN contribute imperceptibly to the improvement on strength of PLA-PHB, which is due to phase slipping induced by FEN, but FEN can obviously not be regarded as a plasticizer. It is not surprising that the OTR value of EVOH is much lower than that of PLA-PHB with or without FEN because the permeation of gas occurs mainly through the amorphous region (Michaels et al., 1963 ; Mokwena & Tang, 2012). However, the shortcoming of low crystallinity of PLA was overcome in a great extent with the existence of PHB. The addition of FEN has improved the oxygen barrier performance, which may be mainly attributed to the finely merged of FEN in the structure of PLA-PHB that narrow the space. Apart from providing a basic protection, isolation from water and oxygen to food, packaging also functions as a communication media to inspectors and consumers. Therefore, high transparency and colorless would be preferred for food industry. PLA is highly bright while PHB is somewhat yellow, thus the color of PLA-PHB blends differs according to the changing formula. When the proportion of PLA and PHB is 3:1, the blends showed a good transparency, and with the addition of FEN, the degree of haze decreased and transmittance increased, achieving a similar degree to EVOH. All the mechanical property tests underlined that the addition of FEN has modified the properties of PLA-PHB and closely reached that of EVOH.

Release of FEN oil into aqueous food simulants

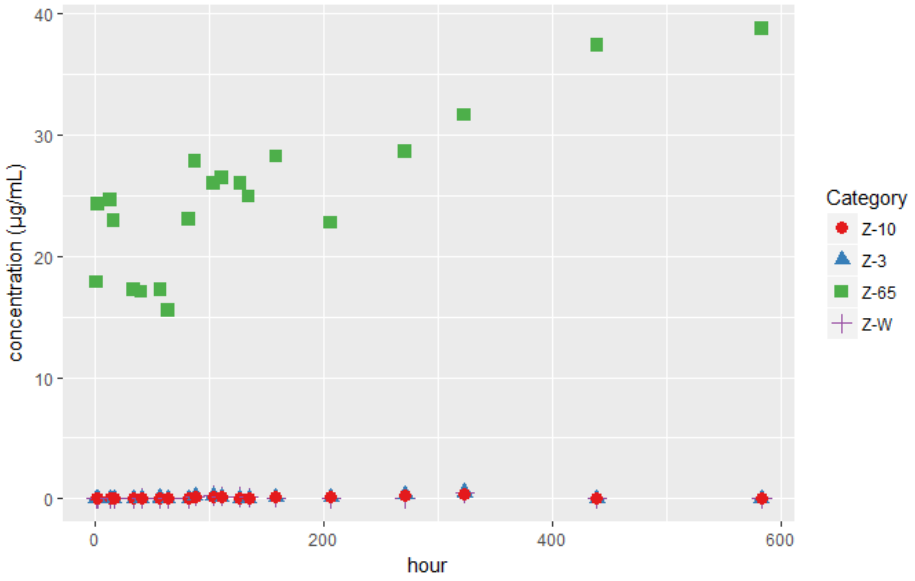

Figure 3. 1 Concentration of FEN oil released to 4 food simulants. ‘Z’ stands for PLA-PHB-FEN film, ‘w’, ‘3’, ‘10’, ‘65’ represent distilled water, 3% acetic acid, 10% ethanol and 65% ethanol respectively.

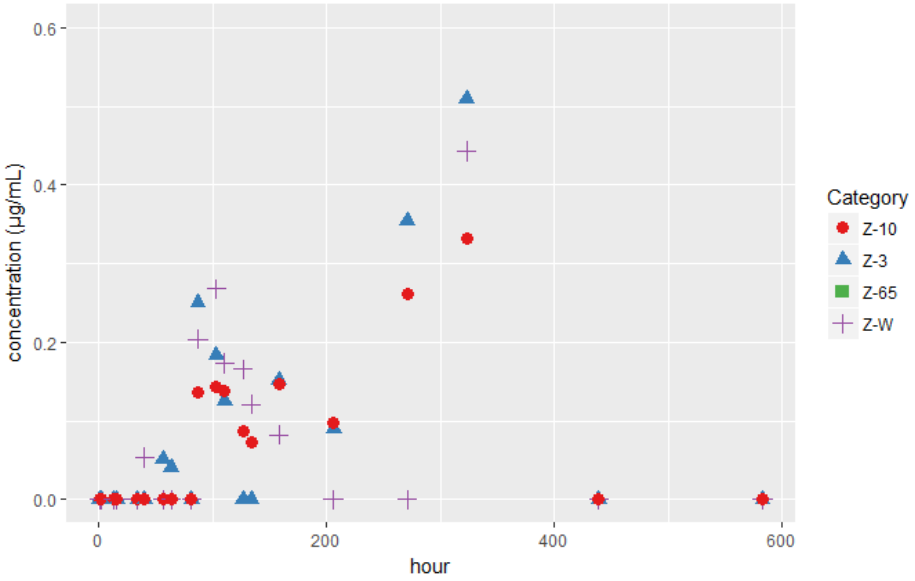

Figure 3. 2 Designated concentration range (0-0.6 µg/mL) of FEN oil released to food simulants.

Prior to determining the amount of FEN oil released into the four food simulants, the calibration curve was first made with a series of appropriate dilutions of raw material, in the meantime, the main ingredient was identified, that is anethol (82.15%).

The release of FEN oil into food simulants is shown in Fig 3.1 and Fig 3.2 PLA-PHB-FEN film in 65% ethanol had the fastest and highest release, going up to 17.89 µg/mL within 0.5 hr., and 38.82 µg/mL at the end of the testing period (583 hr.). Comparing to 65% ethanol, the release into water, acetic acid and 10% ethanol was much slower and smaller. They started to release after 2 to 3 days' immersion, and the maximum concentration were 0.44, 0.50 and 0.33 µg/mL respectively. Subsequently, the dissolved FEN oil gradually evaporated until undetectable. FEN oil prefers to dissolve in ethanol, chloroform and ether, rather than water (Parthasarathy et al., 2008), which indicated that FEN oil would be more applicable on the preservation of oily food.

Antioxidant activity

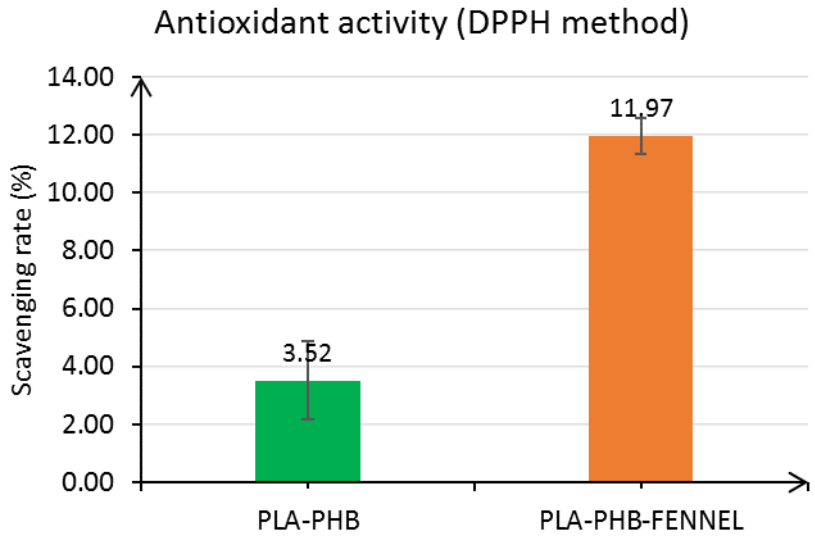

Figure 3. 3 Free radical scavenging rate of PLA-PHB and PLA-PHB-FEN film (n=3; error bar: standard deviation).

Fig 3.3 illustrates the radical scavenging activity of PLA-PHB based film with or without FEN oil. PLA-PHB blank film has a 3.52±1.35% radical cleaning ability while it is 11.97±0.62% for PLA-PHB-FEN film, that is to say FEN oil (anethole) has an improved antioxidant ability but still very weak. Senatore, et al., carried out the antioxidant activity test (DPPH method) on pure anethole and other 6 concentrations of anethole extracted from fresh FEN leaves, turning out that anethole has a weak capacity to catch free radical and does not directly proportional to the concentrations (Senatore et al., 2013).

Bactericidal activity

Table 3. 2 Antibacterial effectiveness on E. coli and Staphylococcus aureus of PLA-PHB-FEN. All values are means ± standard deviations from measurements (n=3).

	Inoculate number (CFU)	Count number after incubation (CFU)	PLA-PHB-FENNEL (CFU)
E. coli	5.33±0.05	6.42±0.13	5.23±0.14
Staphylococcus aureus	5.54±0.02	6.30±0.06	4.94±0.09

Table 3.2 shows the antibacterial and growth inhibition ability of PLA-PHB-FEN film. It reduced the number of E. coli by about 0.1 log and Staphylococcus aureus by 0.6 log, and inhibit the number of growth by 1.2 log and 1.3 log respectively. This result may indicate that anethole in FEN oil has more effects on gram-positive than negative bacteria agreeing with the data reported in (Anwar et al., 2009). And, the data shows a relatively low antibacterial activity, that is related to the release. According to the release test, the concentration of FEN oil is about 0.27 μg/mL in water after a 5-days immersion.

Conclusion

According to the results, the most significant difference between EVOH and PLA-PHB is in OTR, where EVOH is about 100-fold lower than PLA-PHB. It's not weird because EVOH has the lowest OTR among commonly used packaging materials. The OTR of EVOH (EVAL® resin with 27 mol% ethylene) could be over 4 orders of magnitude lower than that of polypropylene, polyethylene, and polystyrene (Mokwena & Tang, 2012). At the same time, with the addition of fennel oil, OTR of PLA-PHB is greatly improved. Therefore, we could reasonably believe that PLA-PHB-FEN won't be the influence factor of excessive oxidation to foods, and will not lead to a large growth of anaerobes and facultative anaerobes as EVOH. Another obvious difference is elongation. The results indicated that EVOH is more able to deform itself to endure external force than PLA-PHB, which is greatly due to their inner property. Thus, we should lay more emphasis on modification with enhanced plasticizers or manufacturing methods. For other mechanical properties, PLA-PHB is nearly comparable to that of EVOH. Focusing on PLA-PHB and PLA-PHB-FEN films, we can see that fennel oil contributes an 8% antioxidant ability which is not significant, but it showed a strong antibacterial ability even with a small amount migration in water. In all, PLA-PHB-FEN is a promising food packaging used for oily foods. Bio-based food packaging could be modified to some extent the same as synthetic packaging and finally they could thoroughly replace synthetic ones.

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Chapter 4: Quality evaluation on oysters packed in EVOH, PLA-PHB and PLA-PHB with Fennel

Abstract

A series of storage tests were applied on Eastern Oysters to verify the effectiveness of active bio-based packaging. The bacterial test showed that the oysters in EVOH and PLA-PHB packaging were getting spoiled on day 12 as the total bacteria count was 6.61±0.21 and 6.88±0.08 log CFU/g respectively while in PLA-PHB-FEN, the number was 7.04±0.18 log CFU/g on day 16. Based on the criterion of 7 log CFU/g rule, we can see that the pH value of oyster samples was 5.98 and 5.96 in PLA-PHB and EVOH while it was 5.79 in PLA-PHB-FEN packaging, and the TVBN value was 12.717±1.781 and 14.726±2.732 mgN/ 100g while it was 14.809±1.184 mgN/ 100g in PLA-PHB- FEN packaging. The dominant free amino acids in oyster samples are glutamic acid, histidine, alanine and arginine, accounting for about 92.91% of total amount of amino acids. Oyster samples have less total amount of amino acids in PLA-PHB-FEN than other two packaging, indicating that there were less bacteria activity due to the inhibitory action of fennel. Therefore, we concluded that this biodegradable packaging is effective to extend the shelf-life of oyster samples for 2 to 3 days with the function of fennel oil.

Introduction

Oysters are the most abundant harvested shellfish in the world (National Marine Fisheries Service, 2007). They are rich in proteins and amino acids, zinc and vitamins, but low in fats. Because of their special juicy, briny taste, they are mostly eaten in raw. It has been reported that New Yorkers averagely has two meals of oysters per week and consumed 500,000 bushels of oyster per season in the early 1900s while the people of New Orleans consumed 750,000 bushels of oysters per year during the same time period (MacKenzie Jr, 1996).

Besides fresh shucked oysters in restaurants and bars, jarred oysters are also very welcome for cooking because they are space saving for transportation and time saving. Generally, refrigeration is the only process applied to this kind of oysters, then the shucked oysters could be stored for around 8 days when initial number of bacteria count is about 3.2 log CFU/g (Cao et al., 2009).

Various preservation techniques are used to extend shelf-life of shucked oysters, including low-temperature pasteurization, high pressure treatment, irradiation, chemical preservation and so on (Jakabi et al., 2003 ; Costa et al., 2014 ; Prapaiwong et al., 2009 ; Mahmoud, 2009). However, problems coming with high efficiency cannot be ignored, such as the changing of appearance and texture when treated with high pressure; excessive additives stayed on the surface; unknown changes caused by irradiation.

Antimicrobial packaging is now a popular method used to prolong the shelf-life of foods. There are a couple of ways to add antimicrobial agents to food packaging such as placing sachets/pads containing volatile antimicrobial agents into packages, coating antimicrobials onto polymer surface, immobilizing antimicrobials to polymer by ionic or covalent bonds, and blending antimicrobial agents with polymers directly while extrusion (Appendini & Hotchkiss,

2002). Among these methods, blending antimicrobial with polymer becomes more attractive due to the less additive contacting with foods, and controlled release of antimicrobial compounds.

Antimicrobial packaging has been studied to apply on many foods, such as meat, button mushroom (Quintavalla & Vicini, 2002 ; Qin et al., 2015) and so on, nonetheless, the application on seafood and oysters is seldom reported. In this study, we aimed to explore the usage of bio-based antimicrobial packaging on prolonging the shelf-life of oysters.

Materials and methods

Freshly shucked oysters (Crassostrea virginica) were supplied by Bon Secour Fisheries, Inc., wildly collected at the Gulf Coast, Alabama, and delivered to the laboratory overnight. Refrigeration was the only process to which they have been subjected. These oysters were then selected and packed in a 4 °C walk-in refrigerator. The samples were divided into three lots for three kinds of packaging. Oysters were packed with a sealer sucking out redundant air. Each three oysters were packed in one package for the texture analysis, pH value and total volatile basic nitrogen determination, and free amino acids analysis. And, to avoid the contamination form other operations, one piece of oyster used for the microbial test was individually packed, this combination was named '3+1'. At each time interval (0, 4th, 8th, 12th, 16th day), about two '3+1' of each kind of packaging were used for quality tests.

Storage test on oysters

1. Chemical tests

Three pieces of oysters, the total weight around 40g, were used for pH value, total volatile basic nitrogen (TVB-N) and free amino acids tests.

pH value was determined with a 5.00±0.02g ground meat from whole oysters, which is then diluted with 50 mL deionized water. The mixture was homogenized for 1 min at room

temperature and measured with an ATI orion perpHecT LogR meter (model 370) with a standard polymer pH electrode (12 × 160 mm).

TVB-N, as a main index indicating the freshness of food, was tested under the instruction of Conway Microdiffusion method. A 5.00±0.02g chopped meat was homogenized in 20 mL 4% trichloroacetic acid (TCA) solution for 1 min. After 15 mins standing at room temperature, the mixture was centrifuged for 15 mins at 10000g. Then the Conway diffusion cells (SCIENCEWARE_ Conway Diffusion Cell; Bel-Art, Wayne, NJ, USA) were employed to make the diffusion proceed. 1 mL aliquet of the supernatant and saturated K_2CO_3 (potassium carbonate) solution were placed in the outer annulus at the opposite positions, and 1 mL 1% H_3BO_3 (boric acid) solution was placed in the center cell. Seal the lid with grease and lightly wobble the cell to mix the supernatant and K_2CO_3. During the one-hour store in the 50 °C environmental chamber, the ammonia produced and absorbed by the H_3BO_3. Subsequently the remaining H_3BO_3 was titrated with 0.01 N hydrochloric acid (HCl) solution. A control test was also carried out using 1 mL 4% TCA, instead of sample extract.

Free amino acids were measured using another 0.2g grounded oyster meat together with 1mL 10% TCA. After mixing in a 1.5-mL microcentrifugal tube,the mixture was centrifuged at 10,000 rpm for 15mins at 4 °C using VWR symphony 241 Microcentrifuge (VWR International LLC., United States). Filter the supernatant with 0.45-μm PTFE membrane and transfer it into A 2-mL vial for high performance liquid chromatography (HPLC) detection.

Two packages of each kind of film were used for tests.

Amino acid standard (AAS18) containing 17 amino acids was purchased from Sigma-Aldrich Co. (St. Louis, MO, United States). Borate buffers (5061-3339), o-phthalaldehyde (OPA,

5061-3335) and 9-fluorenylmethylchloroformate (FMOC, 5061-3337) used for pre-column derivatization were ready-made solutions purchased from Agilent Technologies (Logistics Center Americas, DE, United States). Na_2HPO_4, $Na_2B_4O_7•10H_2O$ and NaN_3 used for the mobile phase A were purchased from VWR International LLC. (United States). 1.4 g Na_2HPO_4 and 3.8 g $Na_2B_4O_7•10H_2O$ completely dissolved with 100 mL deionized water was filtered with vacuum filtration systems equipped with 0.2 μm PES membranes (VWR®, United States), followed by dilution at ratio of 1:10 with deionized water. Afterwards, 32 mg NaN_3 was added to prevent the growth of bacteria. The pH value of mobile phase A was then adjusted to 8.2 with HCL and degassed for HPLC process.

Mobile phase B composed of acetonitrile, methanol, and deionized water at the ratio of 45:45:10 (v:v:v) was also prepared.

The free amino acids detection was conducted with the HPLC system (1260 Infinity, Agilent Technologies, CA, United States) with an Agilent ZORBAX Eclipse Plus C18 Columns (25cm × 4.6mm, 5um). And the injector program was written as followed:

Draw 2.5 µL from location ‘Vial 2’ with default speed using default offset
Draw 1 µL from sample with default speed using default offset
Mix 3.5 µL from seat with default speed for 5 times
Wait 0.2 min
Wash needle in location ‘Vial 1’ 3 times
Draw 0.5 µL from location ‘Vial 3’ with default speed using default offset
Mix 4 µL from air with default speed for 10 times
Draw 0.4 µL from location ‘Vial 4’ with default speed using default offset
Mix 4.4 µL from seat with default speed for 10 times
Draw 32 µL from location ‘Vial 5’ with default speed using default offset
Mix 20 µL from air with default speed for 8 times
Inject
Wait 0.1 min
Switch valve to ‘Bypass’

Vial 1, 2, 3, 4, 5 were filled with distilled water, borate acid, OPA, FMOC and distilled water

Microbial test

A portion of 10.0 g oyster meat sampled randomly with 90 mL sterile water were put into either side of a Whirl-Pak™ Filter Bags (Nasco®, United States), pooled and homogenized for 2 minutes. A series of decimal dilutions was carried out and spread on the prepared Plate Count Agar (HiVeg™ Plate Count Agar, HiMedia Laboratories, India), then incubated both aerobically and anaerobically at 37°C for 36 to 48h.

Texture profile analysis

Texture profile analysis was performed with TA. XT2i Stable Micro Systems Texture Analyzer (Stable Microsystems, Surrey, UK). The parameters were set as (Zhang et al., 2016) which was applied on oysters as well. An aluminum cylinder probe TA 30 was employed to perform two successive compression cycles with 5 second elapse, each compress dropped to 50% of the original height of each oyster. The force, work of area and time were given to calculate the hardness, adhesiveness, springiness, cohesiveness, gumminess, resilience, providing a general information about the texture of oysters.

Statistical analysis

Prior to one-way ANOVA testing, means and standard deviations were calculated. Data of all tests were analyzed using one-way ANOVA, and to understand the difference among three packaging and the change during storage, factors ‘Day’ and ‘Category’, corresponding to ‘testing period’ and ‘films’ were tested individually. The R software (version 3.3.3) was used to perform these analyses.

Results and discussion

Changes in total bacteria count (TBC)

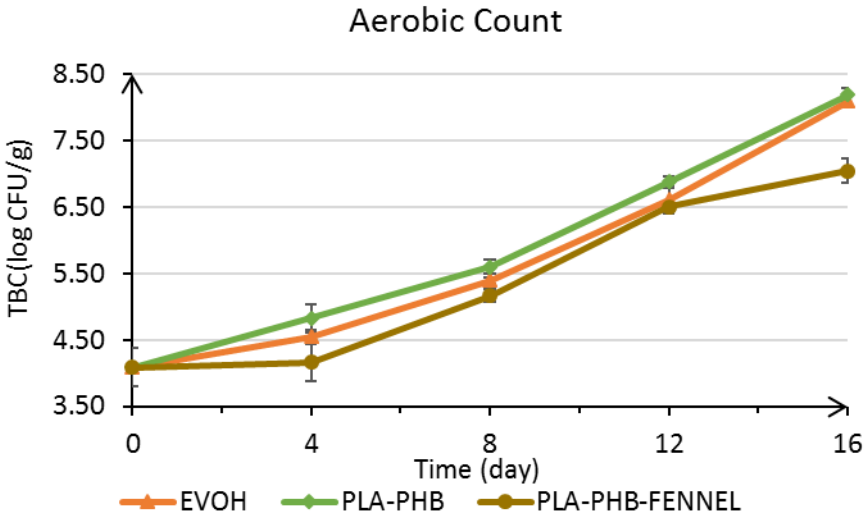

Figure 4. 1 Total aerobic bacteria count of oysters in three packaging. Points and bars stand for the average value and standard deviation of measurements.

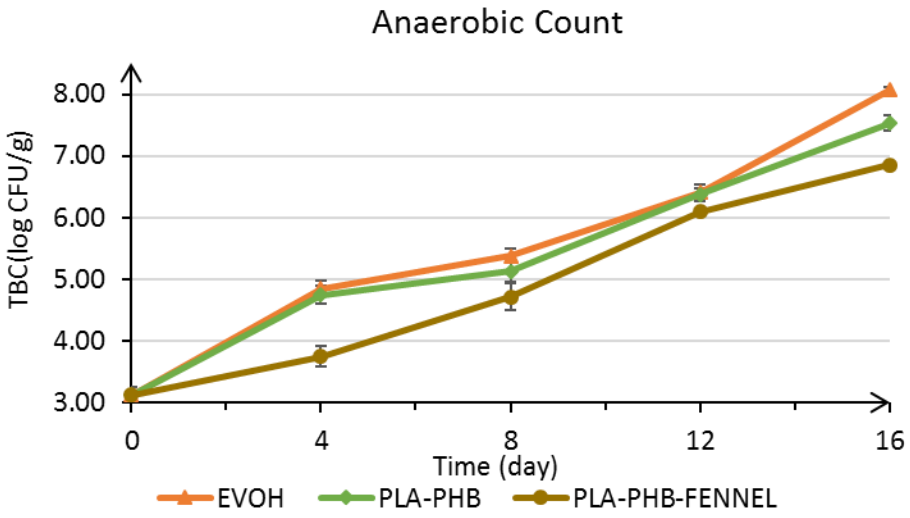

Figure 4. 2 Total anaerobic bacteria count of oysters in three packaging. Points and bars stand for the average value and standard deviation of measurements.

Fig 4.1 and Fig 4.2 show the growth of aerobic and anaerobic bacteria throughout a 16-day storage under 4°C. The initial number of aerobic and anaerobic bacteria is 4.09±0.29 log CFU/g and 3.13±0.13 log CFU/g, following a significant increase in next 16 days (variable

'day', $P<0.05$). The difference of aerobic and anaerobic among three packaging are both remarkable based on the ANOVA analysis (variable 'category', $P<0.05$). In Fig 4.1, it is apparent that oysters in PLA-PHB is easier to spoil than those in EVOH and PLA-PHB-FEN. The growth in EVOH was comparable to PLA-PHB-FEN till Day 12, and then the number went up continuously in EVOH while there was a delay exist in PLA-PHB-FEN, similar result was observed in anaerobic counting, that may due to the accumulated amount of FEN oil released, which is consistent with the release test and Yichao (Ma et al., 2017). In Fig 4.2, little difference can be detected between EVOH and PLA-PHB, that is because EVOH film is a good oxygen barrier, thus anaerobic bacteria grew rapidly, and for PLA-PHB film, it has neither antibacterial ability nor oxygen barrier performance, leading to the rapid growth of anaerobe and facultative anaerobe.

Assuming a microbial criterion of 7 log CFU/g as acceptable, EVOH and PLA-PHB can provide a 12-13 days' preservation for oysters (6.61±0.21 and 6.88±0.08 log CFU/g) while PLA-PHB-FEN can give a 16 days' storage (7.04±0.18 log CFU/g).

Changes in TVB-N

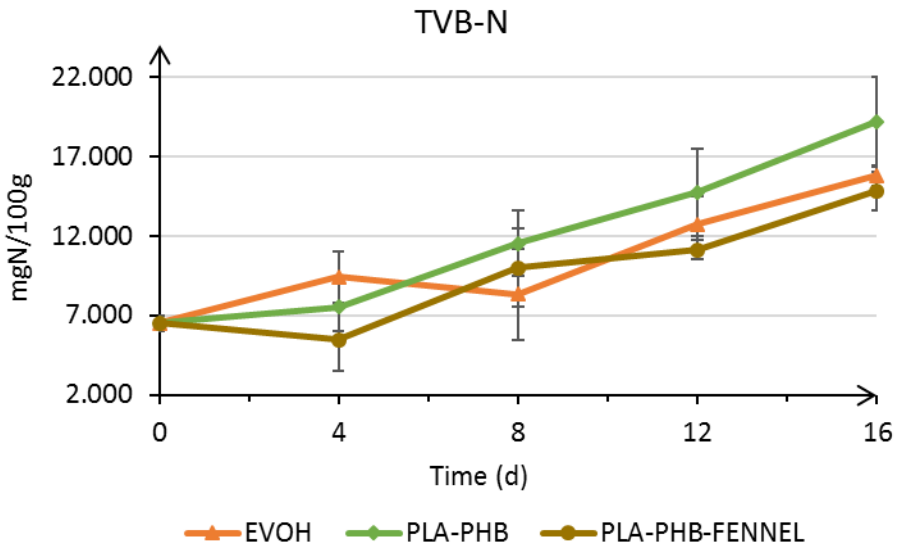

Figure 4. 3 Total volatile basic nitrogen of oysters during the storage with three packaging. Points and bars stand for the average value and standard deviation of measurements.

The change of TVB-N of oysters is shown in Fig 4.3 The initial amount of TVB-N in fresh-shucked oysters was 6.510 ± 0.490 mg N/100g. With the pack of three packaging, the TVB-N value experienced a mild increase. The TVB-N of EVOH and PLA-PHB-FEN was quite lower than PLA-PHB. These three packaging were significantly different from each other, at the same time, the change detected by testing periods within one film was also significant (P<0.05). The production of TVB-N is caused by bacterial decomposition of trimethylamineoxide (TMAO) and amino acid side chains (Haaland & Njaa, 1989). According to the microbe test in this study, at the time that aerobic count reached 10^7 CFU/g, the TVB-N value of oysters in EVOH and PLA-PHB were about 12.717±1.781 and 14.726±2.732 mgN/ 100g respectively (Day 12) while it was around 14.809±1.184 mgN/ 100g in PLA-PHB- FEN packaging (Day 16). Criterion for TVB-N measurement ranges from 20 mgN/ 100g to 30 mgN/ 100g (Kim et al., 2002 ; Cao et al., 2009 ; Suvanich et al., 2000), but none of our results reaches the level. This

phenomenon is also reported in other researches on oysters (Zhang et al., 2016 ; Suvanich et al., 2000 ; Cao et al., 2009), which indicates that TVB-N can be used to predict the trend of spoilage or compare same samples with different treatments, but it is not a good quality indicator or the criterion should be built up relying on different species**.**

Changes in pH value

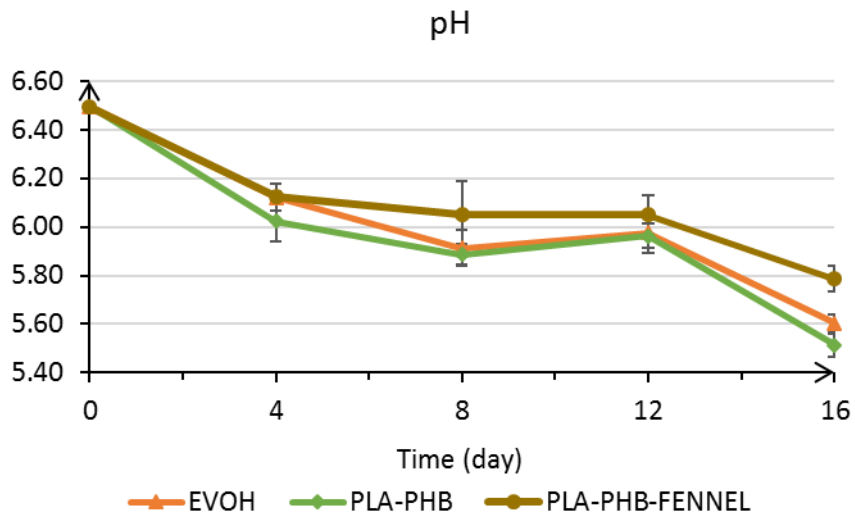

Figure 4. 4 pH value of oysters during the storage with three packaging. Points and bars stand for the average value and standard deviation of measurements.

Fig 4.4 shows the pH value of oysters throughout the storage. The pH of fresh-shucked oysters was 6.50±0.01, which is consistent with (Zhang et al., 2016) and then it had a sharp decrease among all samples. The decrease may due to the lactic acid generated from glycogen as well as the liberation of inorganic phosphate due to the enzymatic degradation of ATP (Sikorski, 1990). Then the pH value maintained stable with a lightly decrease, followed by a remarkable decrease from Day 12 to Day 16 resulted from the large number of bacteria. Generally, in the later storage period, the decomposition of nitrogenous compounds will lead to an increase in pH in the flesh. Nevertheless, based on the result of this study, that the pH value kept going down

without going up is corresponding to the less production of TVB-N. Significant difference was detected in both intra-and inter-group (P<0.05). On the day that the oysters were judged as spoiled, the pH value is 5.98±0.08 and 5.96±0.05 (Day 12) for EVOH and PLA-PHB, and 5.79±0.05 (Day 16) for PLA-PHB-FEN.

Amino-acid composition analysis

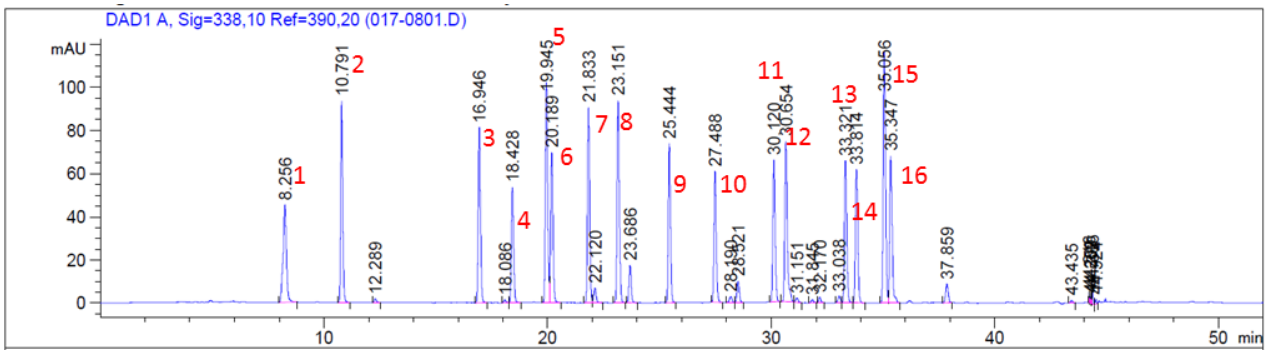

Figure 4. 5 HPLC spectrum for amino acids standard

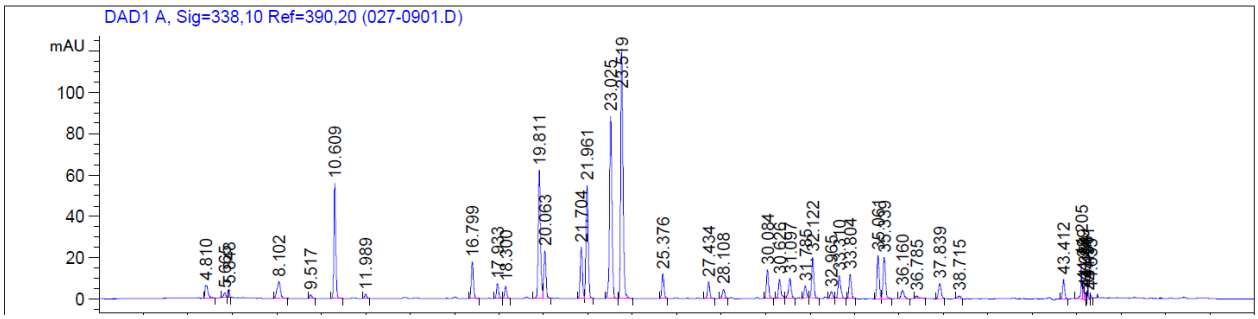

Figure 4. 6 HPLC spectrum for amino acids of oyster sample

Amino acids standard consists of 16 free amino acids were identified in Fig 4.5, they are 1. Aspartic acid (Asp); 2. Glutamic acid (Glu); 3. Serine (Ser); 4. Glutamine (Gln); 5. Histidine (His); 6. Glycine (Gly); 7. Arginine (Arg); 8. Alanine (Ala); 9. Tyrosine (Tyr); 10. Cysteine (Cys); 11. Valine (Val); 12. Methionine (Met); 13. Phenylalanine (Phe); 14. Isoleucine (Ile); 15. Leucine (Leu); 16. Lysine (Lys), all of them are in L-enantiomorphs form. Comparing HPLC

spectra of oyster samples with that of standard, the amino-acid composition of oyster samples was recorded and calculated.

Glu, His, Arg and Ala account for 23.38%, 18.54%, 9.14% and 41.85% respectively of total amount of amino acids, that can be regarded as dominant FAAs in eastern oysters, therefore, contributing to the flavor of oysters. Glu provides a unique and complex flavor to foods, especially seafood, that is umami. Ala is the only amino acid with a truly sweet-tasting in L form, and is about 0.54% sweetness as compared with sucrose solution (Solms, 1969). Opinions on the contribution of His to flavor varies a lot, most of researches group it as flat in taste (Solms, 1969), however, Simidu et al. cited by (KONOSU, 1979) found the more palatable species contain more free His. Arg is another major constituent of shellfish, although it does not contribute to sweet or bitter, it is still considered to be the active taste components in abalones, scallops, and short-necked clams (Tanimoto et al., 2013).

Different from these four main amino acids, the amount of Tyr, Cys, Met, Asp, Val and Ile is quite little to even no detectable.

Analyzing the data from ‘Day’ (testing interval) aspect, we studied the change of each amino acid during 16 days in three films. The results show that all packaging had significant differences on Gly, Lys, Ser and Phe ($P<0.05$), mainly because value ‘0.00’ exists. During the storage, the amount of Gly, Lys and Ser from non-detectable (0.00) to detectable while that of Phe decreased to not detectable. Considering about the flavor, the increase of sweet ‘Gly’ and the decrease of bitter ‘Phe’ may contribute to a milder taste of oysters. Besides, the change of Glu was also remarkable ($P<0.05$). The amount initially decreased and then turn to an increase. In terms of other three dominant amino acids, the amount of His and Arg experienced a slight decrease followed by a gentle increase while that of Ala remained at a same level.

Analyzing the data from 'Category' (three films) aspect, we compared the change of each amino acid on each sampling date. The difference of each amino acid among three films is not obvious, however, when we add up all amino acids, the differences become significant.

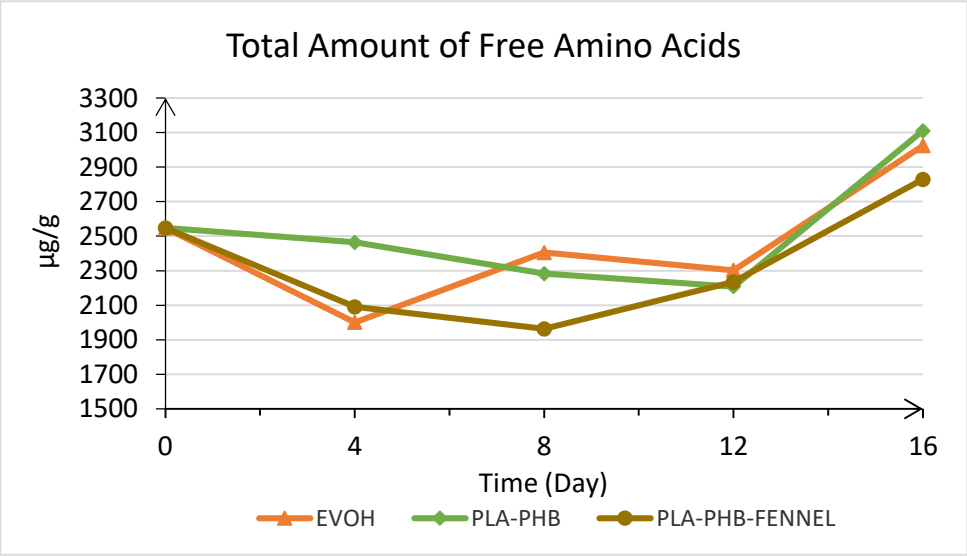

Figure 4. 7 Total amount of free amino acids in oysters packed with three films

Fig 4.7 shows the changes of total FAAs in oysters packed with three films. In the earlier period of storage, there are significant decreases in three packaging, agreeing to the observation by (Tanimoto et al., 2013). Later, the amount of FAAs went up caused by bacteria activity and proteins decomposition. Intuitively, total amount of FAAs in samples packed with PLA-PHB-FEN kept at the lowest level, indicating less bacterial activity took place in it, which is consistent to the results of total aerobic bacteria count in this research, therefore, producing less volatile basic nitrogen. However, the changing trends of total amount of FAAs are not completely same to that of total aerobic count, which may due to a delay between bacterial activity and change of amino acids.

Changes in texture profile analysis (TPA)

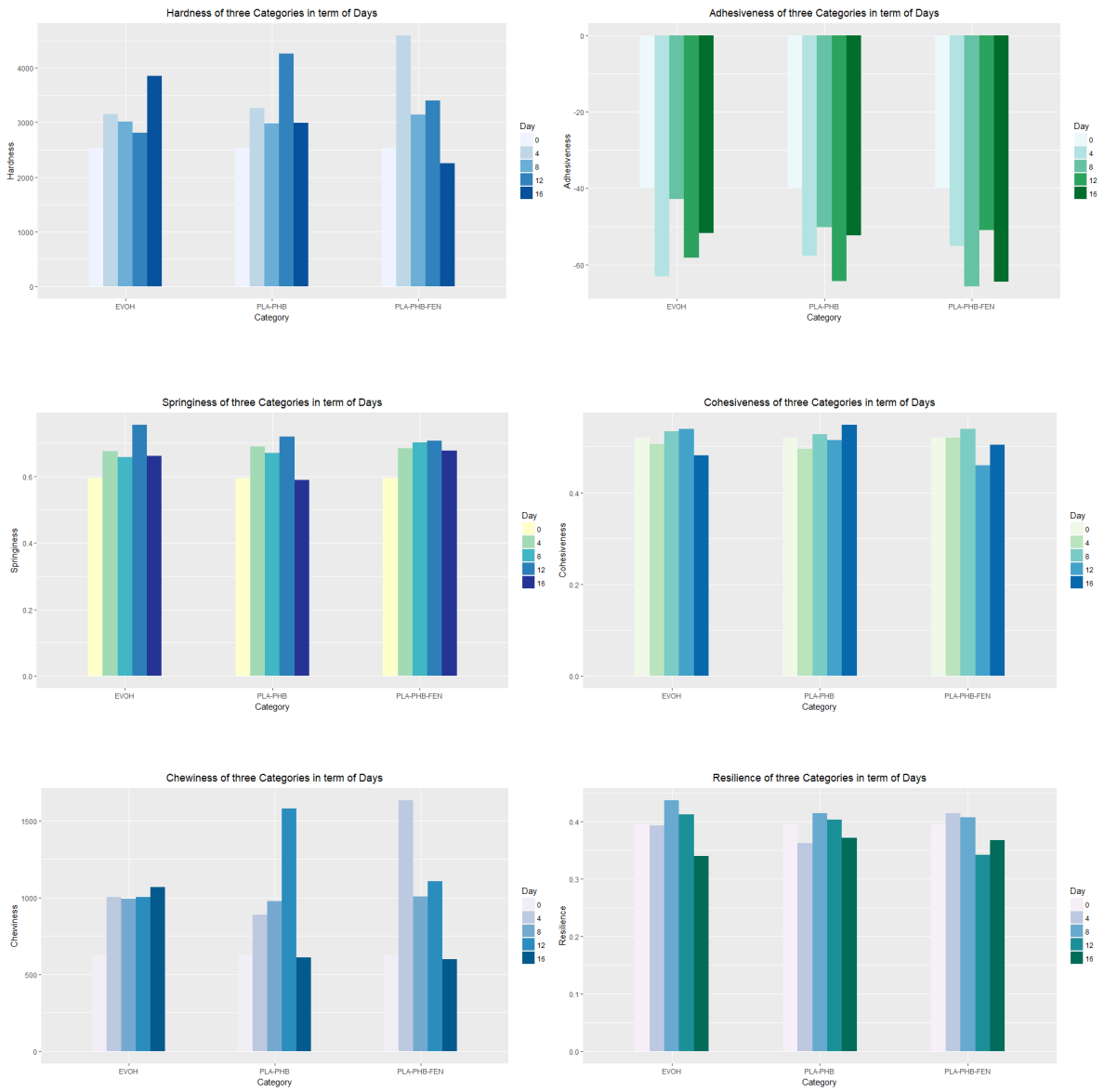

Figure 4. 8 Texture change of oysters in three packaging during storage

Texture profile including hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience of oysters in three packaging films were showed in Figure 4.8. It is noticeable that the parameter 'Adhesiveness' is measured as negative because it refers to the work when testing probe is leaving the sample after first compression. Statistical analysis indicated that oysters in three packaging are significantly different on hardness, adhesiveness and chewiness (P<0.05), and only the adhesiveness and springiness of oysters in PLA-PHB were observed to be different

with respect to ‘Day’. Which means even though the oysters were inedible after a 16 days’ storage according to the bacteria count, their texture didn’t change observably. This suggests that the TPA on oysters cannot be used to judge the difference and change effectively in this study.

Conclusion

When applied on oyster samples, the active film showed the ability to reduce about one log CFU/g on both aerobic and anaerobic bacteria at the end of testing period. It is noteworthy that fennel oil presented a higher antimicrobial ability under an anaerobic condition since that most of foods are preferred to be stored in a vacuum or low-oxygen packaging. Besides, the change of pH and TVB-N were also obviously inhibited with the existence of fennel oil. Although the shelf-life of oysters was only extended for 2-3 days, we could reasonably anticipate a longer extension of PLA-PHB-FEN used on oily food (lipid content of oysters account for 2-4%) as fennel oil is prone to release to fatty simulants.

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