DEVELOPMENT OF ANTIMICROBIAL PROTECTIVE FOOD COATING MATERIALS FROM EDIBLE ALGINATE FILMS

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ABSTRACT

DEVELOPMENT OF ANTIMICROBIAL PROTECTIVE FOOD COATING MATERIALS FROM EDIBLE ALGINATE FILMS

Consumer interests in high quality, healthy, convenient and safe food continue to increase, presenting food processors with new challenges to which functional edible coating and film concepts offer potential solutions. The interest in the research of edible film which has many advantages and applications has increased during last decade. There is a particular interest in the use of antimicrobial biopreservatives in edible films and to increase food safety without application of chemical preservatives. In this study, we have developed antimicrobial or protective edible films by incorporation of antimicrobial enzyme lactoperoxidase or protective cultures (*Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus plantarum*) into alginate films, respectively. The main objective of this research was to increase food safety by using lactoperoxidase or lactic acid bacteria incorporated into alginate films. The results obtained in the study showed that in reaction mixtures, the lactoperoxidase system has antimicrobial activity against *E. coli*, *L. innoc*ua, and *P. fluorescens*. The developed lactoperoxidase incorporated antimicrobial films also reduced the total microbial load of a selected seafood during cold storage. The lactic acid bacteria, used in edible films for the first time, also successfully incorporated into alginate films. The bacteria showed sufficient stability in alginate films and at surface of red meat during cold storage. The results of this study clearly showed the good potential of using lactoperoxidase and lactic acid bacteria incorporated alginate films in food packaging. The developed films can be used in antimicrobial packaging or protective packaging. However, further studies are needed to show the beneficial effects of developed films on different food systems.

ÖZET

YENİLEBİLİR ALGİNAT FİLMLER KULLANILARAK ANTİMİKROBİYAL-KORUYUCU ETKİSİ OLAN GIDA KAPLAMA MATERYALLERİNİN GELİŞTİRİLMESİ

Tüketicilerin kaliteli, sağlıklı, kolay hazırlanabilir ve güvenli gıdalara gösterdikleri yüksek talep, gıda üreticilerini yeni çözüm arayışlarına yöneltmektedir. Fonksiyonel yenebilir fimler ve kaplamalar kullanılarak gıdaların kalite ve güvenliğinin arttırılması konsepti üreticilerin bu arayışlarına potansiyel çözümler getirmekte ve birçok avantajlar sağlanmasına ve uygulamalara olanak sağlamaktadır. Örneğin, üzerinde en yoğun olarak çalışılan konulardan birisi de antimikrobiyal etkiye sahip biyoprezervatiflerin yenebilir filmlerle birlikte gıdalarda kullanılması ve gıda güvenliğinin kimyasal koruyucular uygulanmadan arttırılmasıdır. İşte bu çalışmada laktoperoksidaz ve koruyucu kültürler kullanılarak (*Lactobacillus delbrueckii* subsp. *lactis* ve *Lactobacillus plantarum*) sırasıyla antimikrobiyal ve koruyucu etkisi olan alginat filmler üretilmiştir. Bu araştırmanın temel amacı belirtilen biyoprezervatifler kullanılarak gıda güvenliğinin arttırılmasıdır. Elde edilen sonuçlar üretilmiş olan laktoperoksidaz içeren alginate filmlerin oluşturulan deneysel reaksiyon karışımlarında *E. coli*, *L. innoc*ua, and *P. fluorescens* bakterilerine karşı antimikrobiyal etkiye sahip olduğunu göstermiştir. Geliştirilmiş olan laktoperoksidaz içeren alginat filmler seçilmiş bir deniz ürününde de uygulanmış ve bu ürünün depolanması sırasında toplam canlı bakteri sayısında kayda değer bir azalma sağlanmıştır. Diğer yandan, literatürde ilk kez gerçekleştirilen laktik asit bakterilerinin alginat filmlere ilave edilmesi çalışması da başarıyla gerçekleştirilmiştir. Kullanılmış olan laktik asit bakterileri gerek filmler içerisinde gerekse uygulandıkları seçilmiş gıda olan kırmızı et yüzeyinde yeterli stabiliteyi göstermektedirler. Bu çalışmada elde edilmiş olan sonuçlar laktoperoksidaz ve laktik asit bakterisi içeren alginat filmlerin gıda paketleme uygulamalarında kullanılabileceğini göstermiştir. Geliştirilmiş olan filmler antimikrobiyal paketleme veya koruyucu paketleme amacıyla kullanılabileceklerdir. Ancak, geliştirilen filmlerin çeşitli gıdalarda denenmesi amacıyla ilave çalışmalar gerçekleştirilmesi gerekmektedir.

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CHAPTER 1

INTRODUCTION

Foods may undergo physical, chemical and microbiological deterioration during storage and distribution. Their stability is dependent on a function of changes in their components (proteins, lipids, carbohydrates, and water) owing to environmental and processing factors (Cha and Chinnan 2004). An adequate selection of food packaging and edible coatings could prevent food quality loss by providing barrier and protective properties (Labuza 1996). Various kinds of antimicrobial substances can also be incorporated to improve its functionality since these substances could limit or prevent microbial growth on food surface (Han 2000).

Due to the occurrence of foodborne outbreaks, researchers have been focused on antimicrobial packaging technologies to inhibit microbial growth in the foods while maintaining quality, freshness, and safety. The antimicrobial chemicals incorporated into packaging materials contain organic or inorganic acids, metals, alcohols, ammonium compounds or amines (Appendini and Hotchkiss 2002, Suppakul et al. 2003). However, consumers expect to have natural, more stable and safe products (Hugas et al. 1998, Lemay et al. 2002, Leray et al. 2002). This can be achieved by mild processes without using chemical preservatives. Due to consumers' concerns about chemicals, there is a particular interest in food industry to use natural biopreservatives such as antimicrobial enzymes and bacteriocins for antimicrobial packaging instead of chemical agents (Devlieghere et al. 2004, Padgett et al. 1998). Biopreservatives could be incorporated into biodegradable films since the incorporation of these agents into plastic films is not suitable in terms of denaturating effects of processing methods. The biodegradable film formation is materialized under mild conditions and they are consumable (Suppakul et al. 2003, Appendini and Hotchkiss 2002, Han 2000, Quantavalla and Vicini 2002). They are generally made from proteins, polysaccharides and lipids used alone or together (Khwaldia et al. 2004). Various biopreservatives could be incorporated into edible films to broaden their antimicrobial properties. Park et al. (2004) incorporated lysozyme into chitosan in order to increase its antimicrobial activity. Min et al. (2005) investigated the antimicrobial effects of lactoferrin, lysozyme and lactoperoxidase system together with edible whey protein films. Natrajan et al.

(2000) examined the use of protein and polysaccharide based films containing nisin against Salmonellae on fresh broiler skin. Siragusa and Dickson (1992) added organic acids to calcium alginate gels to develop a potential for raw meat decontamination.

In this study, lactoperoxidase (LP), one of the most important enzymes used in the food packaging as an antimicrobial agent, was used as a biopreservative in alginate films. It catalyzes the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2). This is called lactoperoxidase system (LP system) and this system has a broad antimicrobial activity. The effect of the LP system is either bactericidal or bacteriostatic, depending on the bacterial species, but it has also virucidal, fungicidal, and tumoricidal activity in vitro. This antimicrobial activity is caused by some oxidizing agents such as hypothiocyanite anion ($OSCN^-$), hypothiocyanous acid (HOSCN) and other short-lived antimicrobial products. These agents can oxidize essential sulphydryl groups (-SH) in metabolic enzymes, thereby inhibiting bacterial growth (Kamau et al. 1990, de Wit et al. 1996, and Jacob et al. 2000). The addition of thiocyanate and/or hydrogen peroxide to milk to activate naturally occurring LP system is used to improve microbial quality of milk and cheese (Seifu et al. 2000a, Jacob et al. 2000, Pakkanen and Aalto 1997, Seifu et al. 2000b). The addition of LP and other components of this antimicrobial system to thermally processed skim milk, meat and vegetable products in order to prevent the development of pathogenic bacteria has also been studied (Elliot et al. 2004, Touch et al. 2004, Kennedy et al. 2000). Recently, the LP was incorporated into edible whey protein films to determine the antimicrobial effect of these films on different microorganisms and also on smoked salmon samples. The results revealed that edible whey protein films incorporating LP had good potential for using as antimicrobial packaging (Min and Krochta 2005, Min et al. 2005a, Min et al. 2005b).

Another biopreservative used in this study was the protective cultures. Protective cultures are considered as food grade bacteria (Rodgers 2001), which are also termed as antagonistic cultures (Devlieghere et al. 2004). Lactic acid bacteria constitute the majority of protective cultures (Rodgers 2001a and 2001b). The aim of their antagonistic activity is to inhibit other microorganisms through competition for nutrients and/or production of primary or secondary metabolites (Vermeiren et al.2004, Devlieghere et al. 2004). Some of the compounds released by the bacteria have low molecular weight, such as lactic acid, H_2O_2, CO_2, alcohols, phenyllactic acids, cyclic dipeptides and short or medium chain fatty acids (Rodgers 2003, Vereecken and Van Impe 2002, Schnürer and Magnusson 2005) and some have high molecular weight,

amongst which are polysaccharides and bacteriocins (Vereecken and Van Impe 2002). Bradholt et al. (1999) showed that lactic acid bacteria might be used as protective cultures to inhibit growth of *L. monocytogenes* and *E. coli* in cooked meat products. Budde et al. (2003) investigated lactic acid bacteria in vacuum-packed meat products and evaluated their potential as protective cultures in vacuum-packed meat products. Rodgers et al. (2004) also studied the growth of non-proteolytic *C. botulinum* and protective cultures, toxins and bacteriocin production in a liquid medium at refrigeration temperatures.

In this study, we have developed protective antimicrobial edible alginate films incorporating antimicrobial enzyme lactoperoxidase or protective cultures, *Lactobacillus delbrueckii* subsp. *lactis* and *L. plantarum*. The main objective of this research was to increase food safety by the use of lactoperoxidase or lactic acid bacteria incorporated alginate films. The formation of lactoperoxidase mechanism in alginate films and use of these films to increase food safety was achieved. It was also observed that the protective cultures incorporated into edible films could be utilized as an alternative method for food preservation.

CHAPTER 2

PACKAGING

2.1. Packaging

Packaging is today indispensible vehicle to maintain the quality of foods during storage, transport and handling. It is necessary to protect food products from outside influences and damage, to hold the food products together, to provide consumers with ingredient and nutritional information. In addition, it helps minimizing the use of chemical additives and reducing the impact of packaging waste on the environment (Marsh and Bugusu 2007, Schou et al. 2004).

Active packaging is a kind of packaging aiming at changing the condition of packed food in order to extend its shelf-life, improve safety or enhance sensory properties (Quantavalla and Vicini 2002). Major active packaging techniques are concerned with a variety of chemical (chelators, antioxidants, flavors, essential oils, etc.) or antimicrobial compounds (bacteriocins, organic acids, lysozyme, etc.); gas (i.e. ethylene, carbon dioxide, oxygen, nitrogen, etc.) scavengers or emitters; humidity absorbers or controllers; aroma absorbers or emitters; or active enzyme systems.

2.1.1. Antimicrobial Food Packaging

The presence and growth of pathogenic or spoilage organisms spoil fresh foods easily. This is also a reason of increase in the risk of foodborne illnesses. There are some traditional methods of preserving foods such as thermal processing, drying, freezing, refrigeration, irradiation, modified atmosphere packaging, and adding antimicrobial agents or salts. Unfortunately, some of these techniques cannot be applied to some food products, such as fresh meats and ready-to-eat products (Quantavalla and Vicini 2002).

Considering the fact that microbial contamination of these foods occurs primarily at the surface, due to post-processing handling, attempts have been made to improve safety and to delay spoilage by use of antibacterial sprays or dips. Different

chemicals such as organic or inorganic acids, metals, alcohols, ammonium compounds or amines can be incorporated into the packaging materials as antimicrobials. On the other hand, direct surface application of antibacterial substances onto foods have limited benefits. The active agents are neutralized on contact or diffuse rapidly from the surface into the bulk of food. This may result in partial inactivation of the active substances by product constituents such as lipids and proteins (Hoffman et al. 2001, Pranoto et al. 2005, Quantavalla and Vicini 2002). Therefore, it is clear that this type of protection has limited effect on the surface microflora.

The increased demand for safe and minimally processed fresh produce has intensified the researches on antimicrobial packaging. It is a promising form of active food packaging. The use of packaging containing antimicrobial agents could be more efficient. Slow migration of the agents from the packaging material to the surface of the product localizes the functional effect at the food surface. Moreover, they remain at high concentrations for extended periods of time (Hoffman et al. 2001, Pranoto et al. 2005, Quantavalla and Vicini 2002).

2.1.1.1. Types of Antimicrobial Packaging

The antimicrobial food packaging acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packaged food or packaging material itself. It can take several forms as discussed below.

2.1.1.1.1. Addition of Sachets/Pads Containing Volatile Antimicrobial Agents into Packages

The most successful commercial application of antimicrobial packaging has been sachets. They can be enclosed loose or attached to the interior of a package. There are three forms: oxygen absorbers, moisture absorbers, and ethanol vapor generators (Appendini and Hotchkiss 2002).

Oxygen Absorbers: These system consumes oxygen from the package headspace and oxygen that enters through the package wall. Oxygen scavenging is an effective way to prevent growth of aerobic bacteria and molds in dairy and bakery products (Suppakul et al.2003). Oxygen absorbers cause oxidation of either iron or

ascorbic acid. The former is more common. By using iron powder, it is possible to reduce the oxygen concentration in the headspace to less than 0.01%. One drawback of iron-based scavengers is that they normally cannot pass the metal detectors. As an alternative to the iron-based absorbers, ascorbic acid, ascorbate salts or catechol can be utilized. However, their uses are not widespread (Robertson 2006).

Moisture Absorbers: Liquid water can be accumulated in packages as a result of respiration in horticulture produce, melting of ice, temperature fluctuations in food packs with a high equilibrium relative humidity, or drip of tissue fluid from cut meats and produce. The purpose of these scavenging system is to lower water activity of the product. Thereby, it suppresses the growth of microorganisms on the foodstuff. Pouches containing NaCl and desiccants have been successfully used for moisture control in a wide range of foods, such as tomato, cheeses, meats, chips, nuts, popcorn, candies, gums and spices (Suppakul 2003).

Ethanol Vapor Generators: In ethanol generating system, sachets containing encapsulated ethanol release its vapor into the packaging headspace. Therefore, it maintains the preservative effect on the product (Suppakul 2003). It is only effective in products with reduced water activity ($a_w < 0.92$), and retards molds in bakery and dried fish products (Appendini and Hotchkiss 2002).

2.1.1.1.2. Incorporation of Volatile and Non-Volatile Antimicrobial Agents Directly into Polymers

Antimicrobials may be incorporated into polymers in two ways: (1) incorporation of the agents when the polymer is in the melt form, (2) incorporation of antimicrobial substances into solvents containing the polymer (solvent compounding). Thermal polymer processing methods, extrusion and injection molding, may be utilized with thermally stable antimicrobials. Silver substituted zeolites, for example, can resist very high temperatures (up to 800^{o}C). For heat sensitive antimicrobials like enzymes and volatile compounds, solvent compounding may be more appropriate method for their incorporation into polymers (Appendini and Hotchkiss 2002).

The antimicrobial substances incorporated into packaging material may be volatile or non-volatile. Antimicrobial packaging materials must contact the food surface if they are non-volatile. Hence, antimicrobial agents can diffuse to the surface.

At this point, surface characteristics and diffusion kinetics become crucial. The diffusion of antimicrobial from the film should occur at a suitable rate (in other words controlled release is expected which means release in a slow manner, not a rapid release) because the surface concentration can be maintained at minimum inhibitory concentration at the food surface.

Packaging systems that release volatile antimicrobials have also been developed. These are chlorine dioxide, sulfur dioxide, carbon dioxide and allylisothiocyanate. They can penetrate the bulk matrix of the food. In this case, the polymer does not need to contact the product (Appendini and Hotchkiss 2002).

2.1.1.1.3. Coating or Adsorbing Antimicrobials to Polymer Surfaces

There are some antimicrobials which cannot tolerate the temperatures. They have also been used in polymer processing (extrusion and injection molding). For this reason, heat sensitive antimicrobials are often coated onto the material after forming or are added to cast films. Cast edible films, for example, have been utilized as carriers for antimicrobials and applied as coating onto packaging materials and/or foods. An example includes nisin incorporated zein coatings for poultry (Appendini and Hotchkiss 2002). Another example is the LDPE films coated with a mixture of polyamide resin in propanol and a bacteriocin solution (Suppakul et al. 2003). The most important point is that the location of coating may influence the migration of antimicrobial substance (Fig. 2.1)

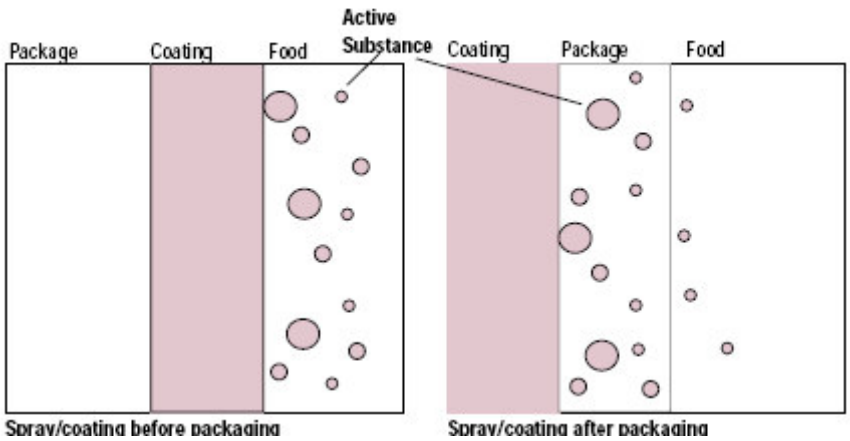


Figure 2.1. The migration of antimicrobial agent from the coating material. (Source: Han 2000)

2.1.1.1.4. Immobilization of Antimicrobials to Polymers by Ion or Covalent Linkages

This type of immobilization occurs with the presence of functional groups on both the antimicrobial and the polymer. Peptides, enzymes, polyamines and organic acids are potential examples for antimicrobials with functional groups. There are also some examples of polymers used for food packaging that have functional groups. These are stated in Table 2.1.

Table 2.1 Antimicrobials covalently/ionically immobilized in polymer supports. (Source: Appendini and Hotchkiss 2002)

Functional support	Antimicrobials
Ionomeric films	Benomyl
	Benzoyl chloride
	Bacteriocin
Polystyrene	Lysozyme
	Synthetic antimicrobial peptides
Polyvinyl alcohol	Lysozyme
Nylon 6,6 resins	Lysozyme

2.1.1.1.5. Use of Polymers that are Inherently Antimicrobial

Some polymers have an inhibitory antimicrobial effect. They have been used in films and coatings. Examples of cationic polymers are chitosan and poly-L-lysine. These polymers promote cell adhesion owing to the fact that charged amines interact with negative charges on the cell membrane. As a result, this reaction causes leakages of intracellular constituents.

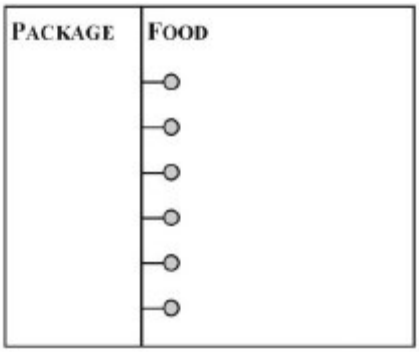


Figure 2.2. Immobilization antimicrobial agents onto food packaging materials.
(Source: Quintavalla and Vicinni 2002)

2.1.1.2. Antimicrobial Packaging Systems

Most food packaging systems consist of either a package/food system or a package/headspace/food system. In a package/food system, the packaging material contacts with solid, or low-viscosity or liquid food without headspace. Individually wrapped ready-to-eat meat products, sous-vide cooked products and deli products can be given as an example for this kind of packaging. The main phenomena involved in this system is the diffusion of antimicrobials incorporated into the packaging to the food and partitioning at the interface (Fig. 2.3) (Quintavalla and Vicinni 2002).

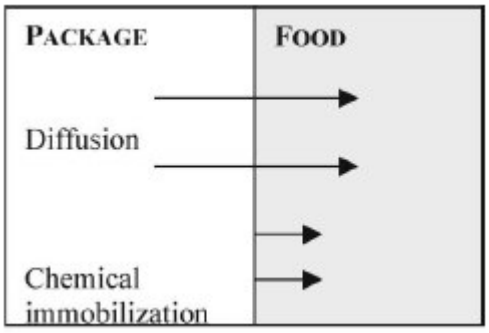


Figure 2.3. Packaging/Food systems.
(Source: Quintavalla and Vicinni 2002)

In a package/headspace/food system, a volatile active substance migrates through the headspace and air gaps between the package and the food. Flexible packages and cups, are good examples of this system.

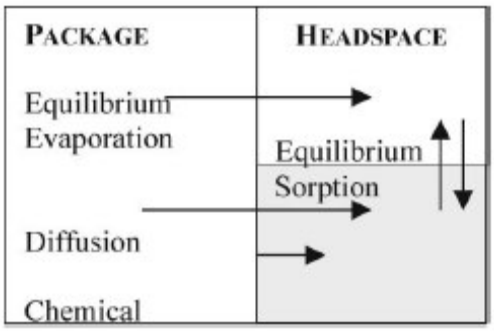


Figure 2.4. Package/Headspace/Food Systems.
(Source: Quintavalla and Vicinni 2002)

2.1.1.3. Important Factors Considered in the Manufacturing of Antimicrobial Films

There are several factors to be considered in the design or modelling of the antimicrobial film or package since an antimicrobial agent may affect the inherent physico-mechanical properties of the package.

2.1.1.3.1. Process Conditions and Residual Antimicrobial Activity

The effectiveness of an antimicrobial agent used for the packaging may be changed during film fabrication, distribution, and storage. The chemical stability of an incorporated antimicrobial substance is likely to be influenced by the extrusion conditions (high temperatures, shearing forces and pressure). Moreover, some processes (laminating, printing, and drying) may affect the antimicrobial compounds which could be lost during storage. All these parameters should be considered in evaluating the effectiveness of an antimicrobial agent (Quintavalla and Vicinni 2002, Suppakul 2003).

2.1.1.3.2. Characteristics of Antimicrobial Substances and Foods

Food components significantly influence the effectiveness of the antimicrobial substances and their release. Physico-chemical characteristics of food , including water activity, pH and acidity, could change the activity of antimicrobial substances. For example, the pH of food alters the ionisation of most active chemicals, and could

influence the antimicrobial activity of organic acids and their salts. The food water activity could change the microflora, antimicrobial activity, and chemical stability of the incorporated active ingredients (Quintavalla and Vicinni 2002). Moreover, aerobic microorganisms can use headspace oxygen. Therefore, oxygen present in the headspace may influence the microbial growth (Suppakul 2003).

2.1.1.3.3. Chemical Interaction of Additives with Film Matrix

Polarity and molecular weight of an additive are of great significance during incorporation of additives into a polymer. Antimicrobials with low polarity are compatible with non-polar polymeric materials. Furthermore, the molecular weight, ionic charge and solubility of different additives influence their diffusion rates in the polymer. For example, amongst ascorbic acid, potassium sorbate and sodium ascorbate in calcium-alginate films at 8,15 and 20^{o}C, the most diffused agent was the ascorbic acid. These was due to the ionic states of the additives (Suppakul 2003).

2.1.1.3.3. Storage Temperature

Storage temperature can influence the antimicrobial activity of chemical preservatives. Generally, the migration of the active agents in the film/coating layers can be accelerated by the increased storage temperature. In contrast, refrigeration slows down the diffusion rate. The temperature conditions during production and distribution have to be taken into consideration in order to determine the residual antimicrobial activity. (Suppakul 2003, Han 2000).

2.1.1.4. Testing the Effectiveness of Antimicrobial Packaging

There are several methods to assess the antimicrobial effectiveness of films on microorganisms. The most common techniques are discussed in the following sections (Appendini and Hotchkiss 2002).

2.1.1.4.1. Minimum Inhibitory Concentrations (MIC) Method

The minimum inhibitory concentration is the lowest concentration of an antimicrobial in a polymer that still inhibits the growth of a particular microorganism. This can be determined using tube dilution procedures. In this method, films containing different dosage of antimicrobials are incorporated into tubes containing growth media inoculated with the target microorganisim. The growth of microorganism is monitored as a change in turbidity. By this way, the break point of the antimicrobial in the polymer that prevents growth of the microorganism *in-vitro* can be determined.

2.1.1.4.2. Dynamic Shake Flask Test

This method provides more detailed confirmation on antimicrobial kinetics. Liquid media (buffer, growth media, or foods) are seeded with the target microorganisms and the antimicrobial polymer. The flasks are incubated with mild agitation at appropriate temperatures. Samples are taken over time and enumerated in order to observe the reduction in microbial growth rate.

2.1.1.4.3. Agar Plate Test

An antimicrobial containing film is placed on a solid agar medium containing the test microorganism. The agar plates are incubated until growth is visible. A clear zone surrounding the film is explained as antimicrobial diffusion from the film and this diffusion of the antimicrobial from the film causes an inhibition of growth of target microorgansim. Lack of growth under a film may show inhibition. However, suitable controls must be included. This may be because of simple restriction to oxygen. The agar plate tests method simulates wrapping of foods. It may suggest what can happen when films contact contaminated surfaces. To make this method quantitative the diameter of clear zones can be measured. Another possible uses of this method is to inoculate the target microorgansim on an antimicrobial containing film. As known, post-processing surface contamination is a major issue for food industry. This method may suggest what can happen when microbial contamination occurs on films or coatings contacting foods (Min and Krochta 2005).

CHAPTER 3

EDIBLE FILMS AND COATINGS

3.1. Edible Films and Coatings

Edible films and coatings have become popular in the food industry, because they produce less waste, are cost effective, and offer protection after the package has been opened (Cha and Chinnan 2004). They are defined as continuous matrices prepared from proteins, polysaccharides, and lipids (Çağrı et al. 2003). These are thin layers of edible materials and are formed on the surface of a food as a coating or between food components. They are natural and biodegradable substances, can be consumed along with the food, can provide additional nutrients, can enhance sensory characteristics and may include antimicrobials (Ryu et al. 2002).

During 12^{th} and 13^{th} century, in China, oranges and lemons were dipped in wax in order to slow water loss (Hardenberg 1967). During 15^{th} century, Yuba, the first-standing edible film, was developed from soymilk in Japan (Debeaufort et al.1998). In the 16^{th} century, "larding", coating food products with fat, was used to prevent moisture loss in foods in England, for example, the edible protective coating was applied on meats to prevent shrinkage (Krochta et al. 1994, Debeaufort et al. 1998). In the 19^{th} century, nuts, almonds, and hazelnuts were coated with sucrose to prevent oxidation and rancidness during storage (Debeaufort et al.1998). The more important application of edible films and coatings until now is the use of an emulsion made of waxes and oil in water that was spread on fruits to improve their appearance, such as their shininess, color, softening, onset of mealiness, carriage of fungicides, and to better control their ripening and to retard the water loss.

The main purposes of the films applied on foods are: (Multon 1996, Krochta et al. 1994)

- To provide protection against humidity and/or oxygen
- To limit surface desiccation
- To delay microbial spoilage of the surface
- To maintain water activity in heterogeneous foodstuff

- To control transfer of solutes, pigments, aromas,... etc. in heterogeneous foodstuff
- To prevent absorption of brine, osmotic dehydration of syrup, or frying oil by the foodstuff
- To improve the mechanical properties to facilitate handling on the manufacturing line or during storage and to reduce spoilage and provide structural integrity of a food product
- To protect individual items on foodstuffs
- To fulfill a surface which does not stick or which is not oily
- To prevent the sticking together of small portions
- To improve or modify the color, aroma, or flavor of foodstuffs
- To trap flavor during manufacture and storage.

Foods are described as: " All raw, partially treated or treated substances used for human nutrition and feeding" by Debeaufort et al. (1998). From this point of view, edible films and coatings can be classified as foods. On the other hand, edible packagings do not provide a significant nutritional value to the coated food. Therefore, we should consider them more like an additive than an ingredient. It is clear that it all depends on the application of the edible packaging. It can also be employed to improve the nutritional quality of the food, and so be qualified as a food ingredient. As food components, edible films and coatings usually have to be as tasteless as possible in order not to be detected during the consumption of the edible-packaged food product. Consequently, if we focus on them as a packaging and a food component, edible films and coatings have to fullfill some requirements: they should have good sensory qualities, high barrier and mechanical efficiencies, enough biochemical, physico-chemical and microbial stability, free of toxics, and safe for health, simple technology, low cost of raw materials and process, and be nonpolluting.

3.2. Functionality of Edible Films and Coatings

Edible packagings have functional properties; which are selective and active properties. Selective properties of edible films and coatings are illustrated in Figure 3.1. In most cases, they can be used as water barrier efficiency in order to retard the surface

dehydration of fresh (meat, fruits, and vegetables) or frozen products. For example, coatings could delay the water absorption inducing the caking in food powder or the loss of crispness in dried cakes. The control of gas exchanges (O_2, CO_2, ethylene), in particular oxygen, allows better control of the ripening of fruits or to significantly reduce the oxidation of oxygen-sensitive foods and the rancidity of polyunsaturated fats. Organic vapor transfers can be prevented, which enables aroma compounds retain in the product during storage. Moreover, by the prevention of the transfers, solvent penetration is not allowed in foods, which can involve toxicity or off-flavors. The penetration of oil during frying and of sucrose or sodium chloride during osmotic dehydration can be limited by an edible film. For example, one of the more interesting applications of edible films and coatings is the reduction of water migration in a pie because of their use inside a composite food to control mass transfers between the different compartments of the product.

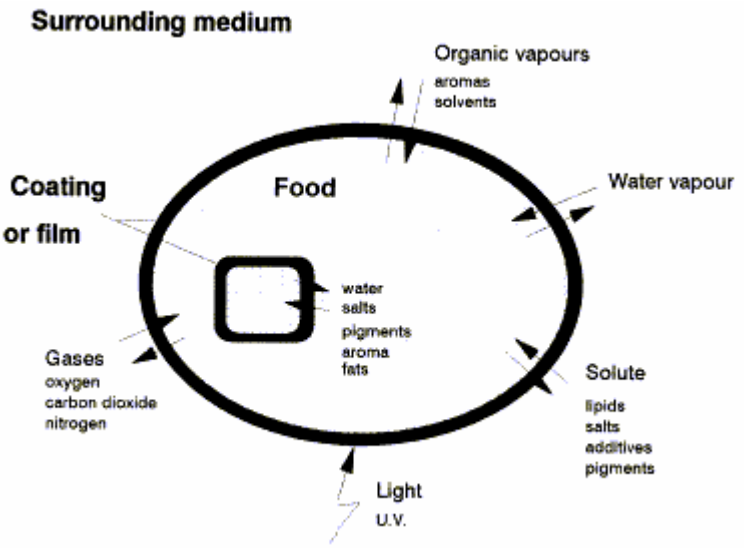


Figure 3.1.Selective functions of edible films and coatings.
(Source: Debeaufort et al. 1998)

As seen from the figure above, the film can also reduce the effect of light and UV light that concerns radical reactions in foods. This property can be improved by incorporating pigments or light absorbers. Therefore, they are active when films are carriers or used for encapsulation of food additives or ingredients. Edible packagings can improve mechanical properties of food to facilitate handling and carriage. Sensorial characteristics such as color, shininess, transparency, roughness or sticking can be

improved. The films and coatings enable to protect or separate small pieces or food portion for individual consumption.

3.3. Film Components

Components of edible films and coatings can be classified into three groups: hydrocolloids, lipids, and composites. Below each type of edible films were discussed in the following sections.

3.3.1. Hydrocolloid Films

Hydrocolloid films can be used in applications where barrier to oxyen, carbon dioxide, and lipids are required (Krochta et al. 1994), but they are sensitive to moisture and show poor water vapor barrier properties. They have suitable mechanical and optical properties (Ryu et al. 2002), and for this reason it makes them useful for strengthening the structure of the foodstuff. Hydrocolloids can be grouped into two classes as carbohydrates or proteins according to their composition (Krochta et al. 1994).

3.3.1.1. Carbohydrate Films

Polysaccharides are nontoxic and widely available. They also have selective permeablity to CO_2 and O_2, and hence retard the respiration and ripening of many fruits and vegetables by limiting the availability of O_2. Polysaccharide-based films have a hydrophilic nature. For this reason, they are a poor barrier to water vapor. The poor water vapor barrier property allows for the movement of water vapor across the film, thus, preventing water condensation that can be a potential source of microbial spoilage in horticulture commodities (Cha and Chinnan 2004). Namely, the coating which has a fairly high water content acts by losing water before the product.

3.3.1.1.1. Starch

Starch is very biodegradable and cost effective, but is also very hydrophilic. Their mechanical properties are generally inferior to synthetic polymer films. When a plasticizer such as water is incorporated, starch exhibit thermoplastic behavior (Cha and Chinnan 2004). Starch is composed of amylose and amylopectin. It is primarily derived from cereal grains, potatoes, tapioca, or arrowroot. Amylose is responsible for the film-forming capacity of starches. Amylose, the linear fraction of starch, is known to form a coherent and relatively strong, freestanding film, in contrast to amylopectin films, which are brittle and noncontinuous. Starch-based coatings containing potassium sorbate have been applied to extend the storage life of strawberries. The formulations with potassium sorbate have not only reduced the microbial count, but also extended the storage life from 14 to 28 days in coated starwberries. In addition, since the water activity (a_w) is critical for microbial, chemical, and enzymatic activities, studies have demonstrated that films can resist the migration of moisture into the meat or poultry during the storage (Cha and Chinnan 2004).

3.3.1.1.2. Alginate

Alginates are salts of alginic acid. They are linear copolymers of D-mannuronic acid and L-guluronic acid and extracted from seaweeds, major structural polysaccahrides of brown seaweeds known as Phaeophyceae (Hui 1991). There are some seaweeds used as commercial production, including *Macrocystis pyrifera*, *Laminaria hyberborea*, *Laminaria digitata*, and *Ascophyllum nodosum*.

Alginic acid is a linear (1->4) linked polyuronic acid containing three types of block structures:

- poly-β-D-mannopyranosyluronic acid (M) blocks
- poly-α-L-gulopyranosyluronic acid (G)blocks
- MG blocks containing both polyuronic acids

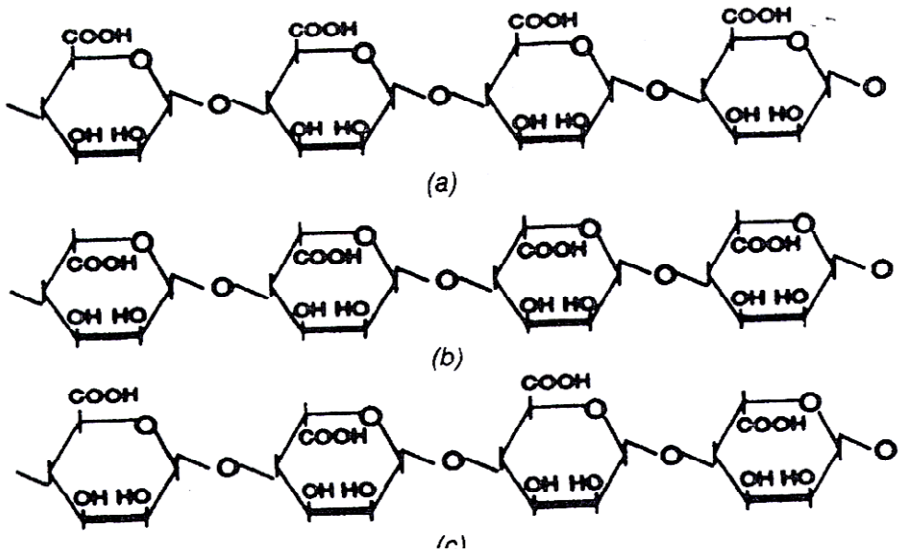


Figure 3.2. Structure of alginates consisting of (a) M block, (b) G block, and (c) alternating M and G blocks (Source: Krochta et al. 1994).

These block regions determine the shape of the polymer which directs how effectively the chains associate during gel formation. The block contents of the alginate are responsible for the different gel strengths of products derived from different seaweeds. Gels prepared with alginates rich in L-gulopyranosyluronic acid tend to be stronger, more brittle and less elastic than those prepared with alginates rich in D-mannopyranosyluronic acid.

Alginates has good film-forming properties. These properties make the alginates particularly useful in food applications. In the procedure of forming an alginate film coating, the food is firstly dipped into a sodium alginate solution and then crosslinked with a solution containing calcium. This calcium solution facilitates for the alginate film forming solution to become gel and makes the alginate polymeric network insolublize (Krochta et al. 2002).

The alginate films have been used for meat, poultry, and fish coating. Calcium alginate coatings decrease moisture loss from the treated foods. It is most likely that the coatings have more water than the coated food product and it behaves as a stock of moisture (Hui 1991, Krochta et al. 1994). A multifunctional calcium alginate coating is used to reduce shrinkage, oxidative rancidity, moisture migration, oil absorption, and seal in volatile flavors of various food products including meat, poultry, seafood, dough products, vegetables, extruded foods, and cheese. Alginate coatings can also provide protection against lipid oxidation of food ingredients (Krochta et al. 1994). Siragusa and

Dickson (1992) tested the use of organic acids immobilized on the surface of inoculated beef tissue to inhibit microbial growth. The researchers showed that the lactic and acetic acids used in immobilized calcium alginate gels inhibited *Listeria monocytogenes* on a lean beef tissue. These acids-alginate gels indicated a promising method for sanitizing and preserving raw meat. Natrajan and Sheldon (2000) examined the use of calcium alginate film including nisin against *Salmonella typhimurium*. They studied the effect of alginate film use for coating broiler drumstick skin samples contaminated with *S. typhimurium* and then coated with the film. The results indicated that the level of reduction in microbial growth was affected by film type and gel concentration, exposure time, and nisin concentration. Pranoto et al.(2005) reported the antibacterial effect of alginate-based edible film incorporated with garlic oil against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus cereus*. The researchers also examined the concentration of garlic oil in the film and concluded that incorporation of garlic oil into alginate film at levels more than 0.2 % led to a significant inhibitory effect on *S.aureus* and *B.cereus*. Cha et al.(2002) studied Na-alginate based antimicrobial films including lysozyme, nisin, grape fruit seed extract and EDTA. The film incorporated with grape fruit seed extract-EDTA showed the strongest inhibitory effect on *Listeria innocua*, *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Microccocus luteus*. Oussalal et al. (2006) evaluated the concentration of the pretreatment used (2% or 20 % $CaCl_2$) and the antimicrobial effect of cinnamon, oregano, and savory essential oils in alginate based films against *E. coli* O157:H7 and *S. typhimurium* on beef muscle. They obtained that after 5 days of storage, *S.typhimurium* was mostly affected by the films containing oregano or cinnamon essential oils regardless of the concentration of the cross-linking agent. In addition, oregano incorporated films were effective against *E.coli* together with the pretreatment of 2% $CaCl_2$. Ogunbanwo and Okanlawon (2006) immobilized bacteriocin produced by *L. brevis* in edible alginate film (1% w/v). In this study, they inoculated the chicken samples with *S .kentucky* AT1 and then examined the shelf-life of refrigerated chicken treated with bacteriocin containing film. They indicated that the immobilized bacteriocin into alginate film could extend shelf-life of samples up to 14 days at refrigerated storage.

3.3.1.1.3. Carrageenan

Carrageenan is a complex mixture of several polysaccharides. As for alginates, the films obtained are transparent, odorless, and very lightly salted in the case of the addition of calcium salts. Their mechanical and protective properties are average, and their applications are restricted to improving the appearance, preventing sticking, and protecting against rancidity of dried products such as dried fruits and protecting frozen meats and fish from surface drying. Some authors searched the reduction of *Staphylococcus aureus* on the surface of an intermediate moisture food by using a coating composed of a carrageenan agarose gel to obtain a surface pH of 0.5 units less than the pH of the rest of the foodstuff (Bureau and Multon 1996).

3.3.1.1.4. Cellulose Derivatives

The modified cellulose ethers, including methyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, and ethyl cellulose, are the basis of many edible film coatings. The methyl celluloses have hydrophilic nature. Therefore, it makes them suitable for components of nonpolar lipid films having moisture barrier properties (Hui 1991). Cha et al (2002) studied the antimicrobial effect of lysozyme incorporating carragenan films. The results showed that these films were not superior than lysozyme incorporated alginate films under the same conditions.

3.3.1.1.5. Pectin Films

Pectins are a group of plant-derived polysaccharides. They work well with low-moisture foods, but they are poor moisture barriers. Pectins are composed of methyl esters of linear chains of 1,4-α-D-galacturonic acid units. During the processing methyl esters may be hydrolyzed, producing low methoxy pectin. The low methoxy pectin have a divalent cation gelling mechanism similar to that of the alginates and carrageenan, and the use of low methoxy pectins as edible coatings is similar to that of the alginates. The food to be coated is first dipped into a solution of low methoxy pectin and then into a solution of low methoxy pectin and then into a solution of calcium chloride. The thickness of the coating can be controlled by the concentration of the pectin or the

viscosity of the pectin solution. These gels have been used as coatings for almonds, candied fruit, and dried fruit (Hui 1991).

3.3.1.1.6. Chitosan

Chitosan is an edible and biodegradable polymer. It is derived from chitin. Chitosan is also an abundant natural polymer available. It forms films without the addition of additives, exhibits good oxygen and carbon dioxide permeability beside excellent mechanical properties. This material not only acts as a chelator in biological system, but also exhibits antimicrobial activity against bacteria, yeasts and molds. However, one disadvantage with chitosan is its high sensitivity to moisture. Quattara et al.(2000) investigated the inhibition effect of chitosan films containing cinnamaldehyde against *S.liquefaciens* and Enterobacteriaceae on bologna. They reported that growth of the microorganisms were delayed or completely inhibited as a result of film application.

3.3.1.2. Protein Films

Proteins may be derived from corn, wheat, soybeans, peanut, milk, or gelatin. They are suitable for coating fruits and vegetables. Protein-based coatings have good barriers to O_2 and CO_2, but not to water. Several protein based edible film discussed in the following section.

3.3.1.2.1. Zein

Zein is a protein extracted from maize or corn. It is only soluble in the mixture of water and alcohol because it is protein. It has good humidity barrier property. This type of protein based edible films has been used commercially in coating formulations of shelled nuts, candy, and pharmaceutical tablets (Bureau and Multon 1996, Hui 1991, Cha and Chinnan 2004). These films are also suitable coatings for dried fruits and intermediate moisture foodstuffs (Bureau and Multon 1996). Mecitoğlu et al.(2005) reported that corn zein films incorporated with partially purified lysozyme showed antimicrobial effect on *Bacillus subtilis* and *Lactobacillus plantarum*. Hoffman et al.(2000) studied the effect of corn zein films impregnated with nisin, lauric acid, and

ethylene diamine tetracetic acid (EDTA) on *L.monocytogenes* and *Salmonella enteritidis.* They found that *L.monocytogenes* could not be detected after 24h exposure to the film combination including lauric acid. The film with EDTA and lauric acid and EDTA, lauric acid, nisin had bacteriostatic effect when the initial inoculum of *S. Enteritidis* was 10^4 CFU/ml was used.

3.3.1.2.2. Whey Protein Isolates (WPI)

Whey proteins account for 20% of total milk proteins. They are characterized by their solubility at pH 4.6. Liquid whey is a by-product of cheese manufacture and is produced in large quantities, but much of this whey is not utilized. Hence, it causes serious waste disposal problems. Whey proteins are water soluble and form hydrophilic edible films (Krochta et al.2002). WPI can form transparent, flexible, colorless, and odorless films that provide excellent oxygen, aroma, and oil barrier properties (Min and Krochta 2005). Çağrı et al.(2001) developed an edible film (pH5.2) from whey protein isolate containing p-aminobenzoic acid (PABA) or sorbic acid (SA). The researchers showed that incorporating 0.5% to 1.5% of SA or PABA into WPI films led to inhibition of *L.monocytogenes*, *E.coli* O157:H7, and *S. typhimurium* DT104 on trypticase soy agar + 0.6% yeast extract.

3.3.1.2.3. Collagen Casings

Collagen is a fibrous protein. The film-forming ability of collagen has been traditionally utilized in the meat industry for production of edible sausage casings. Collagen is manufactured from corium layer of bovine hide which is more than 90% collagen on a dry basis. To obtain casings, the hide coriums is delcalcified and grinded into small pieces. Then, ground collagenous material with acid is mixed to produce a swollen slurry (4-5% solids). The slurry is homogenized and extruded into tubular casings(8-10%). Finally, tha casings free of salts are washed, treated with plasticizer and cross-linking agents and dried (Min and Krochta 2005).

3.3.2. Lipid Films

Lipid compounds contain neutral lipids of glycerides. Glycerids are esters of glycerol and fatty acids and waxes, which are esters of long-chain monohydric alcohols and fatty acids. From this group, acetylated monoglycerides, natural waxes, and surfactants are commonly utilized in edible coatings. Lipids are commonly added to food coatings to impart hydrophobicity. Lipids are mainly used for their efficiency as a water-vapor barrier in edible films. The structure, degree of saturation, chain length, physical state, shape and dimension of crystal, and distribution of lipids into the film influence the functional properties of the film (Cha and Chinnan 2004). Wax and glycerides are examples of lipid based films and described below.

3.3.2.1. Waxes

Waxes belong to the non-polar lipid class. They are insoluble in bulk water and do not spread to form a monolayer on the surface. Their hydrophobicity is high. There are differences in permeability of wax films. These differences is owing to their chemical composition and crystal type. The waxy skin on fresh fruit and vegetables is applied to reduce dehydration and control the exchange of gases to prolong preservation period (Cha and Chinnan 2004). There are some examples of waxes used for coating, including paraffin wax, carnauba wax, beeswax, candelilla wax, polyethylene wax (Krochta et al. 2002).

3.3.2.2. Glycerides

Monoglycerides are used in edible films as emulsifiers, especially for stabilizing emulsified film and increasing adhesion between two components with different hydrophobicity. Triglycerides are insoluble in bulk water, but will spread at the interface to form a stable monolayer. Water affinity or hydrophobicity of triglyceride depends on its structure. By adding palmitic, stearic, lauric acids, and stearyl alcohols to edible films, the moisture barrier properties are greatly enhanced (Cha and Chinnan 2004).

3.3.3. Composite Films

Composite films can be designed by combining lipid and hydrocolloid elements. By this way, it can decrease the disadvantages of each film. When a barrier to water vapor is desired, the lipid component can serve this function while hydrocolloid component provides the necessary durability. Composite films consisting of a conglomerate of casein and acetylated monoglycerides have been studied by Krochta et al.(1990). These films can be used as coatings for processed fruit and vegetables.

3.4. Film Formation

There are several techniques developed to form edible films. These techniques include coacervation, solvent removal and solidification of melt and are given below.

3.4.1. Coacervation

The principle of coacervation is to separate a polymeric coating material from a solution by heating, altering pH, adding solvents, or changing the charge on the polymer concerned (Krochta et al. 1994). In the simple coacervation a hydrocolloid is dispersed in aqueous solution and precipitated or gelified by the removal of the solvent, by the addition of a non-electrolyte solute in which the polymer is not soluble, by the addition of an electrolyte substance inducing a “salting out” effect or by the modification of pH of the solution. Complex coacervation, where two hydrocolloid solutions with opposite charges are combined, inducing interactions and the precipitation of the polymer mixture (Debeaufort et al. 1998).

3.4.2. Solvent Removal

It is generally applied for the production of hydrocolloid edible films. In this process, film-forming material is dispersed in a solvent such as water, ethanol, or acetic acid. These solvents contain several additives including plasticizers, cross-linking agents, solutes,...etc. The film-forming solution is then cast in a thin layer, dried and

peeled from the surface (Çağrı et al. 2003). Solvent removal is of great importance to get solid film formation (Krochta et al. 1994).

3.4.3. Solidification of Melt

Melting followed by solidification is one of the commonly used techniques to produce lipid films. In this method the rate of solvent removal and the cooling rate play an impotant role in the overall properties of the resulting film (Krochta et al.1994).

3.5. Film Additives

Additives may be added to edible films to improve their mechanical, protective, sensory, and nutritional properties. These are plasticizers, antimicrobial agents, antioxidizing agents, colorants,...etc. (Bureau and Multon 1996).

A plasticizer is defined as "a substantially nonvolatile, high boiling, nonseparating substance, which when added to another material changes the physical and/or mechanical properties of that material"(McHugh and Krochta 1994). A plasticizing agent must be compatible with the film-forming polymer. Moreover, its permanence within solvent-polymer system is also of prime importance since this influences the physical and mechanical stability of the film. In addition, the polymer and the plasticizer must also have similar solubility in the solvent used. The plasticizer should not be volatile (or only very slightly volatile) and its degree of retention by the film should be high. Furthermore, other properties, such as its chemical stability, hygroscopocity, color, flavor, and so on, are also more or less important depending on the type of film under discussion. Finally, the concentration of a plasticizer necessarily varies from 10-60% (dry base) according to the nature and type of film and the method of application. The most often used plasticizers in edible films and coatings are mono-, di-, and oligosaccharides (generally glucose syrups or glucose-fructose honey, polyos (principally glycerol and its derivatives, polyethylene glycols, sorbitol), and lipids and its derivatives (fatty acids, monoglycerides and their esters, acetoglycerides, phospholipids, and other emulsifiers) (Bureau and Multon 1996). McHugh and Krochta (1994) used sorbitol as a plasticizer in whey protein isolate solution and reported that sorbitol was more effective than glycerol in those films of equal tensile strength,

elongation, and elastic modulus. However, the workers determined that the sorbitol used in the film had lower oxygen permeabilities. Ghanbarzadeh et al.(2006) studied the effects of sugar plasticizers on the viscoelastic and thermal properties of zein resins. The researchers found that the films containing galactose had better tensile strength and strain at break than films containing fructose and glucose.

Edible films and coatings may be used as means for preservatives (antibacteriological or antioxidizing agents) in order to maintain an effective concentration of these substances on the surface of the foodstuff. The use of edible films as a carrier for preservatives makes it possible to combat the deterioration which occur during the onset of storage (e.g., oxidation of dried fruits or development of microorganisms such as molds, yeasts, or osmophilic bacteria on numerous foodstuffs of medium humidity). The addition of preservatives can be used without destruction of the food. In addition, it also uses low amounts of additives in relation to the weight of the product owing to the fact that a sufficient concentration of the additive is maintained on the surface (Bureau and Multon 1996). Park et al.(2005) incorporated potassium sorbate into chitosan coating solution to enhance preservative effects on the storability of strawberries during storage. Theivendran et al.(2006) evaluated the inhibitory effect of grape seed extract (GSE) and green tea extract (GTE) against *Listeria monocytogenes*. This research demonstrated that the use of soy protein edible film coating containing both nisin and natural extracts was a promising means of controlling the growth and recontamination of *L.monocytogenes* on ready-to-eat meat products. Seydim and Sarikus (2006) determined the inhibitory effects of whey protein based edible films incorporated with essential oils against *E.coli* O157:H7, *S.aureus*, *S.enteritidis*, *L.monocytogenes*, and *L.plantarum*. The researchers observed that the film containing oregano essential oil was the most effective film against these bacteria at 2% level.

Incorporation of antioxidants into packaging materials has also become very popular since oxidation is one of the problems influencing the food quality (Güçbilmez et al. 2007). An antioxidant agent can prevent enzymatic (lipogenase) and non-enzymatic peroxidation of essential fatty acids (Hortmann and Meisel 2007). In the same way, films and coatings may serve to maintain high concentration of colorants, flavors, spices, herbs, acids, sugars, salts,...etc. This may give the foodstuff a strong surface taste and nutritional enrichment without destruction of the food (Bureau and Multon 1996).

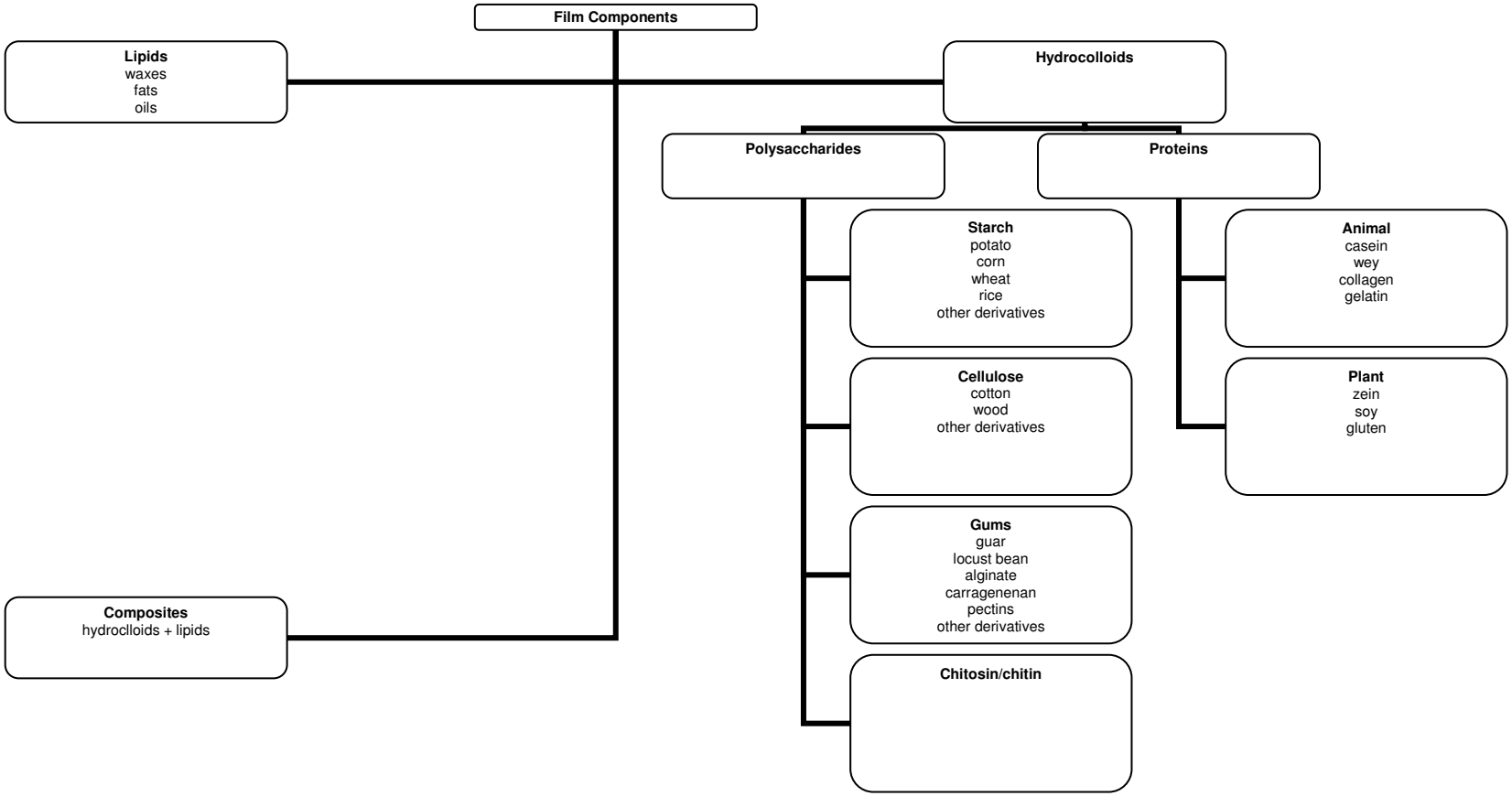


Figure 3.3. Film Components

CHAPTER 4

BIOPRESERVATION

4.1. Definition of Biopreservation

Consumers expect to have natural, more stable and safe products (Hugas et al. 1998, Lemay et al. 2002, Leroy et al. 2002). This can be achieved by the use of mild processes without utilizing chemical additives. It is clear that these additives damage the product's preservative-free status. They, therefore, are not agreeable with the fresh image (Rodgers 2003, Rodgers 2004, Devlieghere et al. 2004, Schnürer and Magnusson 2005, Osmanoğlu 2005). Recently, the food industry and food research have intensified on natural antimicrobial compounds owing to the consumers' ban against chemical preservatives (Devlieghere et al. 2004).

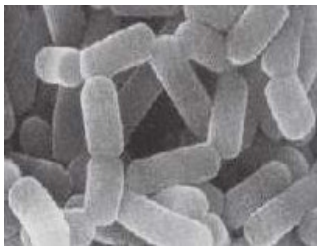
In the early days it was noticed that fermented foods had delicate and refreshing tastes, with an extended shelf life. They also decreased the outbreak caused by foodborne illnesses (Ray 2004). Nowadays, novel technologies such as 'biopreservation' have attracted great attention as means of naturally controlling the growth of pathogenic and spoilage organisms (Mataragas et al. 2003). As it is known, that food additives are widely employed so as to hamper undesired microorganisms and to enhance shelf life. However, biopreservation is a useful device to prevent an overuse of food additives (Schewenninger and Meille 2004). A definition of the biopreservation is as follows: "Biopreservation is a way to prolong storage life and enhance safety by the use of natural or controlled microflora and/or their antimicrobial products."(Rodgers 2003, Hugas 1998, Devlieghere et al. 2004).

As a conclusion, biopreservation is provided by the use of protective cultures and their antmicrobial products or some natural compounds isolated from food. These biopreservatives are briefly explained in the following section.

4.1.1. Protective Culture

Protective cultures are food grade bacteria (Rodgers 2001), which are also termed as antagonistic cultures (Devlieghere et al. 2004). Lactic acid bacteria are

considered as the major group of the antagonistic cultures (Rodgers 2001a, Rodgers 2001b). The objective of their antagonistic activity is to inhibit other microorganisms through competition for nutrients and/or production of primary or secondary metabolites (Vermeiren et al.2004, Devlieghere et al. 2004). Some of the compounds released by the bacteria have low molecular weight, such as lactic acid, H_2O_2, CO_2, alcohols, phenyllactic acids, cyclic dipeptides and short or medium chain fatty acids (Rodgers 2003, Vereecken and Van Impe 2002, and Schnürer and Magnusson 2005) and some have high molecular weight, amongst which are polysaccharides and bacteriocins (Vereecken and Van Impe 2002).



Alcohols	reutericyclin
Caproic acid	Phenyllactic acid
3-hydroxy fatty acids	H_2O_2
Cyclic dipeptides	Reuterin
Organic acids	diacetyl
proteinaous compounds	reutericyclin
CO_2	2-pyrrolidone-5-carboxylic acid

Figure 4.1. Summary of the main antimicrobial compounds produced by LAB (Source: Salmien et al. 2004)

4.1.1.1. Organic Acids

Acetic acid is commercially produced by *Acetobacter aceti.* In general, when the level of acetic acid is 0.2%, it shows bacteriostatic activity, however, bacteriocidal above 0.3%. It is more effective against Gram-negative bacteria. Actually, this effect depends on pH and the bactericidal effect is more definite at low pH (below pH 4.5). It is used as an antimicrobial agent to salad dressings, mayonnaise, and carcass wash (Ray 2004).

Lactic acid is manufactured by some *Lactobacillus* spp. which are able to produce L-lactate (or DL-lactate).The antibacterial effect is pronounced when utilized at 1 to 2 % levels (≥pH 5). At low pH 5, lactic acid can have a bactericidal activity particularly against Gram-negative bacteria (Ray 2004). It is capable of inhibiting the

growth of various food spoilage bacteria, such as Gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae* (Alakomi et al. 2000). However, it may not have an antimicrobial activity on fungi in the food environment (It is usually employed on carcasses) (Ray 2004).

Propionic acid by *Propionibacterium* spp. has fungistic effect in the food environment and also capable of inhibiting the growth of Gram-negative and Gram-positive bacteria. At pH 5 or below, Gram-negative bacteria are more sensitive than Gram-positive ones as used 0.1 - 0.2 % levels,. It is applied to control molds in cheeses, butter, and bakery products and to hamper growth of bacteria and yeasts in syrup, applesauce, and some fresh fruits (Ray 2004).

The antimicrobial property of these organic acids is due to the fact that they are present in the form of undissociated molecules. Their dissociation constants (pKa) for acetic, propionic, lactic acid are 4.8, 4.9 and 3.8, respectively (Ray 2004). They enter the cytoplasmic membrane and result in decrease in intracellular pH and disruption of the transmembrane proton motive force (Alakami et al.2000), eventually cause denaturation of proteins and viability loss (Ray 2004). On the other hand, some studies reported that these weak acids provide antimicrobial action with the combined effects of undissociated molecules and dissociation ions (Ray 2004).

4.1.1.2 Diacetyl

Diacetyl is produced by some lactic acid bacteria during citrate fermentation (Ray 2004, Schnürer and Magnusson 2005). The more diacetyl concentration makes a change in the taste and aroma of the product (Schnürer and Magnusson 2005). Diacetyl (2,3-butanedione) has antimicrobial effects at low pH (Schnürer and Magnusson 2005). Gram-negative bacteria is especially susceptible at pH 5.0 or below. When it is utilized with the combination of heat, it is more bactericidal than when it is used alone (Ray 2004).

Under reduced conditions, acetoin is formed by the conversion of diacetyl. This conversion leads to decrease in antibacterial activity. The antimicrobial effect is likely due to its deactivating property on some important enzymes. The dicarbonyl group (-CO-CO-) makes a reaction with arginine in the enzymes and eventual modification in their catalytic sites (Ray 2004).

4.1.1.3. Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) produced by most lactic acid bacteria is widespread under aerobic conditions (Ray 2004). LAB have flavoprotein oxidases which enable them to produce H_2O_2 in the presence of oxygen (Schnürer and Magnusson 2005). However, they don't have cellular catalase, pseudocatalase, or peroxidase, and therefore the culture release the agent into the environment to avoid its antimicrobial action (Ray 2004). The production of H_2O_2 by LAB can be useful for food preservation and prevention of growth of foodborne spoilage and pathogenic bacteria and pathogens (Villegas and Gilliland 1998).

The synthesis of H_2O_2 by LAB should enable them to prolong the shelf life of some refrigerated foods without altering the acidity of the food (Zálan et al. 2005). H_2O_2 has a strong oxidizing effect on the bacterial cell and on the destruction of basic molecular structures of cellular proteins (Schnürer and Magnusson 2005, Zálan et al. 2005). Bacteriostatic action was shown at 6 to 8 μg/ml H_2O_2 levels, 30 to 40 μg/ml induce bacteriocidal effect. It can also show antimicrobial action against fungi and viruses (also bacteriophages) (Ray 2004).

4.1.1.4. Fatty Acids

Antimicrobial fatty acids can be produced by lipolytic LAB. These acids also make a contribution to the sensory quality of fermented foods. The antimicrobial property can change according to the chain length. For example, caprylic (C_8) acid and longer fatty acids are the most effective, exception of undecanoic, (C_{11}). The researchers found that the growth of yeast cells treated with 10 mM of the fatty acids and monoglycerids was inhibited due to the capric and lauric acid (Schnürer and Magnusson 2005).

4.1.1.5. Phenyllactic Acid

Phenyllactic acid has antimicrobial action only on yeasts and molds at a given concentrations. This metabolite is active in combination with other compounds produced by LAB. In sourdough bread the use of *L.plantarum* delayed the growth of the

mold *Aspergillus niger*, compared to the ose of *Lactobacillus brevis* that did not produce phenyllactic acid (Schnürer and Magnusson 2005).

4.1.1.6. Cyclic Dipeptides

The hydroxylated fatty acids are stronger in terms of antifungal activity than the cyclic dipeptides. These peptides show antifungal activity at given concentrations. (Schnürer and Magnusson 2005).

4.1.1.7. Bacteriocins

Bacteriocins are ribosomally-produced, cationic, and amphiphathic pose α-helical and/or β-sheet structures and can also have thioethers, disulfide bridges, or free thiol groups (Ray 2004). These agents synthesized by LAB have antimicrobial action on closely related species of the bacteria and other Gram-positive spoilage and pathogenic bacteria (Hugas 1998, Alakomi et al. 2000, Osmanoğlu 2003, Devlieghere et al. 2004, Ray 2004,, and Zálan et al. 2005).

The principle of their antimicrobial action is to bind to the surface of the cell wall or the membrane of target organisms, and then form pores in the membrane in order to reduce transmembrane potential and/or pH gradient, namely, its functional destabilization, and provide leakage of cellular materials. Eventually, this causes the death of the cell (Ray 2004, Zálan et al., 2005).

4.1.1.8. Reutericyclin

It is also produced by *L.reuteri*. It is negatively-charged and has highly hydrophobic character. The inhibitory action is rised at higher salt concentration (2%) and low pH. The minimal inhibitory concentration was found to be approximately 0.05-1 mg/L for Gram-negative bacteria and yeasts. It behaves as a proton ionophore, in contrast to other antimicrobial agents which forms pores in the membrane of the target cells. It separates the cytoplasmic membrane, owing to its hydrophobicity, eventually, disrupts the transmembrane ΔpH.

4.1.1.9. 2-Pyrrolidone-5-Carboxylic Acid

Pyroglutamic acid, or PCA, is produced by *Lactobacillus casei* ssp. *casei*, *L.casei* ssp. *pseudoplantarum* and *Streptococcus bovis*. It is also present in fruit, vegetables and grasses. It has an inhibitory activity to *Bacillus subtilis*, *Enterobacter cloacae*, *Pseudomonas putida*, and *Pseudomonas fluorescens*. It is heat stable (20 min at 121 ^{o}C). It is affected by the decrease in pH. Its mechanism of action resembles to that of organic acids (Salmien et al. 2004).

4.1.2. Factors Affecting Protective Culture Performance

The inhibitory effectiveness of a protective culture could vary according to some factors stated below:

4.1.2.1. Temperature Effect

Temperature can affect growth rate, and bacteriocin production of the microorgansims. The protective culture should maintain its viablity in products under refrigerated temperatures. In addition, it should be able to grow and start an antagonistic action at the desired temperatures. Its growth rate should be faster than the food poisoning and spoilage organisms'growth rates. Finally, if the inoculated product is intended for cooking, the heat resistance of the protective culture should be considered in order not to leave the product unprotected after heating. Production rate of bacteriocins and their ability depend on incubation temperatures. Based on temperature, a food poisoning and spoilage organisms can gain sensitiveness to inhibition. For example, psychrotrophic strains of LAB isolated from commercial fresh salads hampered the growth of different pathogens at different temperatures (Rodgers 2001).

4.1.2.2. Protective Culture and Food Poisoning-Spoilage Organisms

A protective culture can be effective against one or several food poisoning-spoilage organisms.The inhibitory substance production rate by a PC and the sensitivity of a FPSO to inhibition depends on the identity of the interactive cultures (Rodgers 2001).

Table 4.1. Application of Protective Cultures to Non-Refrigerated Foods.
(Source: Rodgers 2001)

PC	Temperature (C^o)	Inoculum (cfu/g)	FSPO	Food	Reference
Raw meat,milk, and eggs					
L.delbrueckii subsp. bulgaricus	5	$5x10^8$	*Pseudomonas spp.*	Ground beef	Gilland and Speck (1975)
P.cerevisiae	3	10^9	*Pseudomonas spp.*	Raw poultry	Raccach,Baker, Regenstein,and Mulnox (1979)
P.cerevisiae *Lc. lactis*	7	$5x10^7$	*E.coli o157:H7*	Raw chicken meat	Brashers,Reilly, and Gilliland (1998)
L.delbrueckii subsp. bulgaricus	5,7	10^8	*P.fragi* *Psychrotrophs*	Milk Fresh crab meat	Gilliland and Speck (1975)
P.cerevisiae *L.plantarum*	3	10^9	*Pseudomonas spp.* *S.typhimurium*	Pasteurised liquid whole egg	Raccach and Baker (1979)
Ready-to-eat meals					
E.faecium	3	10^4, 10^7	*L.monocytogenes* *C.botulinum* *C.perfringens* *B.thermosphacta*	Sous vide fish	Smith (1975)
Lc. lactis *L.delbrueckii subsp. bulgaricus*	30	10^6	*C.botulinum*	Chicken a la king	Saleh and Ordal (1955)
L.plantarum ATCC 8014	5,15,25	10^6	*C.botulinum type A,B and E*	Refrigerated can pea soup	Skinner et al. (1999)
L.bavaricus	4,10	10^3,10^5	*L.monocytogenes*	Sous-vide beef	Winkowski et al. (1999)
L.plantarum	4	$4x10^6$	*C.botulinum*	Cured meat	Tanaka,Meske, Doyle, Traisman,Thayer, and Johnson (1985)
P.acidilactici *L.alimentarius* *S.xylous*	7 4	10^{10}	*Micrococci* *Staphylococci* *Brochothix thermosphacta* *Aerobic bacteria* *Pseudomonas*	Cooked ham	Kotzekidou and Bloukas (1996)
L.casei *L.plantarum*	4,8	10^8	*L.monocytogenes* *S.typhimurium*	Ready-to-eat vegetables	Vescovo et al. (1996)

Table 4.1. Application of Protective Cultures to Non-Refrigerated Foods. (cont.)
(Source:Rodgers 2001)

Pediococcus spp.			*S.aureus* *Aeromonas hydrophila*		
P.acidilactici *L.delbrueckii subsp. bulgaricus*	6	$4x10^9$	Background microflora	Tuna and Potato salad	Raccach et al. (1979)
Seafood					
Leuconostoc spp *L.plantarum*	5	10^6, 10^7	*L.monocytogenes* *Yersinia enterocolitica*	Shrimp extracts	Jeppesen and Huss (1993)
Carnobacterium piscicola *L.plantarum*	4	10^8	H_2S-producing bacteria Yeasts and molds	Smoked salmon	Leroi et al. (1996)
Lc.lactis subsp. lactis ATCC 11454 *Carnobacterium piscicola* *L.plantarum*	10	$3x10^6$	*L.monocytogenes*	Cold-smoked salmon	Wessels and Huss (1996)
Others					
L.plantarum *L.mesenteroides* *P.pentosaceus*	15	10^8	*Salmonella* *L.monocytogenes*	Model food slurries	Ernsaw et al. (1993)
Lc. lactis *P.pentosaceus*	10	10^6 10^7	*C.botulinum 17B*	TPGY broth	Rybka-Rodgers (1989)
L.plantarum *Pediococcus spp.*	15	10^8-10^9	*S.typhimurium* *Staphylococcus aureus* *S.aureus* *Aeromonas hydrophila*	Buttered brain heard Infusion Broth	Raccach et al. (1979)
P.acidilactici	10-35	10^6	*C.botulinum*	Chicken salad	Hutton et al. (1991)
L.delbrueckii subsp. bulgaricus	6	$4x10^9$	Background microflora	Tuna and Potato salad	Raccach et al. (1979)

PC: protective culture
FSPO: food poisoning-spoilage organsims

4.1.2.3. Inoculum Effect

Inoculum size affects the production rate and speed of the antimicrobial substances, the sensory quality of the product, and the cost-effectiveness of the method. As seen from the Table 4.1., generally high inoculums (10^6-10^9 CFU/g) are required to show inhibitory activity. The inoculation levels of PC also vary according to the growth

medium, the initial load of other organisms and the identity of the PC used. Excessive microbial load may have a limitation on the growth of the protective culture (Rodgers 2001).

4.1.2.4. Food Effect

Food composition and structure can support or inhibit the protective cultures. For examples, salts, acids, spices, added preservatives have a synergetic effect with PCs. Food can also contain some ingredients, which provide the growth of PCs, such as glucose, yeast extract, biotin,...etc. Indigenous microflora can manufacture its own inhibitory agents on the pathogen involved. The low pH of a product favors bacteriocin production and increase their activity. Growth rates are usually slower in structured food than in liquid environment (Rodgers 2001).

Table 4.2. Factors Effecting Protective Cultures Performance in Non-Fermented Foods. (Source: Rodgers 2001)

Factors	**Possible impacts**
Increase in PC inoculation level	➢ Reduces the time for production of sufficient quantity of inhibitory substance ➢ Excessively high inoculums can prevent culture growth and bacteriocin production
Increase in incubation temperature	➢ Increases the growth rate of PC and FPSO ➢ Can reduce bacteriocin-producing capacity and bacteriocin activity ➢ Can reduce FPSO sensitivity
Choice of a PC	➢ FPSO target range ➢ Rate of inhibiting substance production ➢ Minimum growth temperature ➢ Possible alteration of redox potential ➢ Ability to spoil the product ➢ Survival during processing ➢ Possible health benefits/hazards
Type of food	➢ Growth and inhibitory substances diffusion rate ➢ Indigenous microflora can produce its own inhibitory effect ➢ Antimicrobial substances can have a synergetic effect with the antibiosis ➢ Growth and bacteriocin-production promoting substances can enhance the antagonistic effect

4.1.3. Potential Benefits of PCs

There are some potential benefits of protective cultures (Rodgers 2003, Rodgers 2001). These are as follows:

- Enhancing food safety without imparting the parameters in relation to the process
- Reducing the severity of processing
- Prolonging the shelf life of product, therefore decreasing waste and increasing convenience
- Additive-free preservation
- Natural image
- Temperature-responsive inhibition (against temperature abuse situations)
- Decreasing the energy costs

4.1.4. Application of PCs

Rodgers et al. (2003) reported that bacteriocinogenic lactic acid bacteria could be employed as protective cultures. They found that the inhibitory activity of the selective protective cultures on the different *Clostridium botulinum* strains tested at lower temperatures were high. These protective cultures were Lactobacillus, Lactococcus, Streptoccocus, and Pediococcus species. Rodgers et al. (2004) conducted a research on the inhibition of nonproteolytic *Clostridium botulinum* with lactic acid bacteria and their bacteriocins at refrigeration temperatures. They found that there was no change in the populations of the organisms during incubation at 5^{o}C. On the other hand, the PCs showed inhibitory effect on that pathogen at 10^{o}C , and signified that it was related with the nisin level produced by *L.lactis*. Unfortunately, the sufficient amount of nisin produced by the culture to avert toxin formation by the pathogen was lately accumulated in the media.

Millette et al. (2004) immobilized living cells of lactic acid bacteria in calcium alginate beads and tested for their ability to produce bacteriocins and to inhibit the growth of undesirable organisms. Minor-Pérez et al. (2003) studied that the changes in long-chain fatty acids as well as pH and microbial growth (lactic acid bacteria and

enterobacteria) have an inhibitory effect in pork inoculated with two biopreservative strains, *Lactobacillus alimentarius* and *Staphylococcus carnosus.* Both produce lactic acid, but *S.carnosus* was more efficient in decreasing enterobacteria populations at 20^{o}C. On the other hand, at that temperature, there was a rapid increase of free fatty acids which causes inedibility of the food. In contrast, there was no significant increase in long-chain fatty acid concentration in the samples at 4^{o}C.

Buncic et al. (1997) indicated that on vacuum packaged raw beef (pH 5.3-5.4) and on vacuum packaged emulsion-type sausages (pH 6.4) the amounts of bacteriocin produced in situ by low initial numbers (10^3/g) of the protective strains were inadequate to show listericidal activity at 4^{o}C. In addition, they also explained that high initial numbers of lactic acid bacteria are not desired for the sensory qualities of the products. Vescova et al.(2006) studied that the growth of *Listeria innocua* could be controlled or reduced by the help of antimicrobial-producing lactic acid cultures in vacuum packed cold-smoked salmon. They reported that it could be achieved by the addition of these cultures alone or in combination. Budde et al. (2003) incorporated *Leuconostoc carnosum* 4010 into a vacuum-packaged meat sausage and found that the protective culture decreased the viable number of *Listeria monocytogenes* cells to a level below the detection limit and there was no increase of that pathogenic bacterium during storage at 5^{o}C for 21 days. They suggested that *Leuconostoc carnosum* was useful as a new protective culture for cold –stored, cooked, sliced, and vacuum-packed meat products. Luukkonen et al. (2005) achieved to inhibit the growth of *Listeria* in Edam cheeses from organic milk by the help of a protective culture containing *Lactobacillus rhamnosus* LC705. The protective culture decreased the numbers of *Listeria*, by 0.5 log counts, after 70 days of storage in cheeses. Amézquita and Brashears (2002) suggested that LAB cells during refrigerated storage would prevent the growth of *L.monocytogenes* to infective levels, hence ensuring the safety of the products. They used a bacteriocin producer, *P.acidilactici*, and organic acid producers, *Lactobacillus casei* and *Lactobacillus paracasei*. Their method indicated a potential antilisterial intervention in RTE meats, because it inhibited the growth of the pathogen at refrigeration temperatures without causing sensory changes.

Muthukumarasamy et al. (2003) performed a study to determine the bactericidal effects of the two naturally occurring antimicrobial agents, Allyl isothiocyanate (AITC) and *Lactobacillus reuteri*, on *Escherichia coli* O157:H7 in refrigerated ground beef. The results indicated that the use of AITC with *L.reuteri* was

not effective, but when they are applied alone, the antimicrobial effects were apparent. Saad et al.(2001) conducted a research about the influence of lactic acid bacteria on survival of *E.coli* O157:H7 in inoculated Minas cheese during storage at 8.5^{o}C. They stated that this could be an additional safeguard to well-established good manufacturing practices and hazard analysis and critical control point programs in control of its growth in the cheese.

Kotzekidou and Bloukas (1998) reported that the shelf-life of sliced vacuum-packed frankfurter-type sausage, known as pariza, was prolonged by the inoculation of *L.alimentarius*. However, while suppressing the saprophytic microflora of the samples, *L.alimentarius* could not avert *S.enteritidis* growing with lower growth rates. So, the biopreservation system tested here could not guarantee the safety of sliced vacuum-packed pariza when severe *Salmonella* contamination occurs in this product.

Leisner et al. (1996) controlled the growth of the lactic acid bacteria, *Lactobacillus sake*, which is capable of spoilage of vacuum-packaged meat, by the help of bacteriocinogenic, nonspoiling lactic acid bacterium, *Leuconostoc gelidum* during anaerobic storage at 2^{o}C. It delayed the spoilage by *L.sake* for up to 8 weeks of storage, but chill stored, vacuum-packaged beef inoculated with sulfide-producing *L.sake* developed a distinct sulfide odor within 3 weeks of storage at 2^{o}C.

4.1.5. Antimicrobial Enzymes

Some enzymes that have antimicrobial activity are also used in food industry. These are discussed in the following section.

4.1.5.1. Lactoperoxidase

Research on natural antimicrobials used to enhance in food safety has intensified in recent years. All these studies indicate that consumers will pay more attention to natural antimicrobials than in the past. Lactoperoxidase is one of these agents attracted special interest because of its antimicrobial properties (Yoshida and Ye-Xluyun 1990).

The enzyme lactoperoxidase is a glycoprotein found in mammalian milk, saliva, tears (Jacob 2000). Bovine LP is composed of a single polypeptide chain. It has a molecular mass of 78.431 kDa. LP is a basic protein having an iso-electric point of 9.6

which facilitates its recovery from milk or whey by ion-exchange processes. The major milk proteins have iso-electric points between pH 4.5 and 6.5. This offers possibilities for the recovery and purification of LP by using cation-exchange chromatography at neutral pH. (De Wit and Van Hooydonk 1996).

LP is more active at acidic pH values, but it is less heat stable at this pH range (De Wit and Van Hooydonk 1996). It is fragile and this property has a tendency to increase with increasing purity (Yoshida and Ye-Xluyun 1990).

Table 4.3. Physico-chemical characteristics of LP.
(Source: De Wit and Van Hooydonk 1996)

Characteristics	**Data**
Molecular weight	78.431
Amino acid residues	612
Half-cystine residues	15
Iso-electric point	9.6
Carbohydrate contenet	10%
Iron content	0.07%
Haem structure	Protoporhyrin IX
Folding structure	23% α, 65% β

The LP has an antimicrobial activity when there are thiocyanate and hydrogen peroxide exist in the media. The lactoperoxidase system comprises the enzyme, and two substrates: thiocyanate and hydrogen peroxide (Fonteh 2006)**.** It is a naturally occurring antimicrobial system and has been applied on not only foods but also cosmetics and in clinical applications due to its safety (Thouch et al. 2004). It has a broad antimicrobial activity (Kamau et al. 1990). Its effectiveness is either bactericidal or bacteriostatic, which depends on the bacterial species. In addition, it has also virucidal, fungicidal, and tumoricidal activity in vitro (Pruitt et al. 1982).

LP catalyzes the oxidation of thiocyanate by hydrogen peroxide, yielding short-lived oxidation products, hypothiocyanite ions, and higher oxyacids being mainly responsible for the antibacterial effect of the system (Gaya et al. 1991).

There are several mechanisms for this preservative system and these are given below (Siragusa and Johnson 1989):

- An extended lag period or no growth

- Reduced O_2 uptake
- Reduced lactate production by fermentative organisms
- Inhibition of key metabolic enzymes such as hexokinase, glyceraldehyde-3P-dehydrogenase, and D-lactate dehydrogenase
- Inhibition of glucose uptake
- Cytoplasmic membrane damage with leakage of ions and UV-absorbing material
- Inhibition of nucleic acid and protein synthesis.

The oxidizing products generated by LP system inhibit microorganisms by the oxidation of sulphydryl (SH) groups of microbial enzymes and other proteins. So, it damages the microbial cytoplasmic membrane through the oxidation of SH groups. Finally, this leads to leakage of potassium ions, amino acids, and peptides from microbial cells and eventually causes the death of the cells (Min et al. 2005).

The oxidation of thiol groups (-SH) of cytoplasmic membrane and damage to other cellular elements such as the outer membrane, cell wall or cytoplasmic membrane, transport sytems, glycolytic enzymes and nucleic acids are the results of the LP-catalysed reactions (Touch et al. 2004).

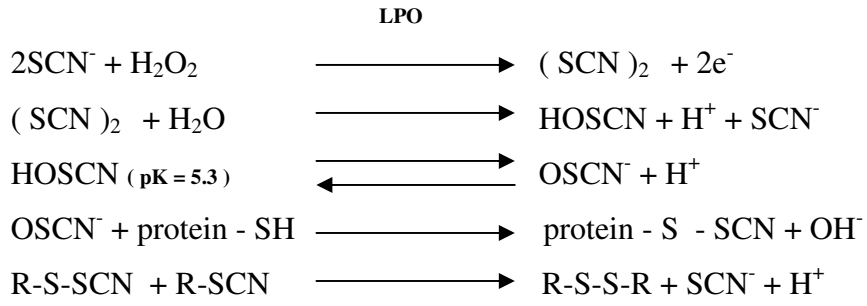


Figure 4.2. Oxidation of protein (enzyme) sulphydryls by lactoperoxidase (LP) catalysed reactions, mediated by products of SCN^- (Source: De Wit and Van Hooydonk 1996).

As mentioned above, this system yields a variety of oxidizing products. The reaction can continue by direct oxidation of SCN^- to $OSCN^-$ or by oxidation of SCN^- via $(SCN)_2$ (thiocyanogen). Among these products, hypothiocyanite ion ($OSCN^-$) is the major inhibitory product at physiologic pH (Siragusa and Johnson 1989, Pruitt et al. 1982).

$OSCN^-$ is in equilibrium with HOSCN (pK 5.3). This indicates that at pH 5.3 (at the pH of maximal LP activity) half is in the form of HOSCN and half is in the form of $OSCN^-$ (De Wit and Van Hooydonk 1996). At low pH, antibacterial action is greater, owing to the ability of the uncharged HOSCN to penetrate microbial membranes and thus to attack functional groups of essential intracellular enzymes (Thomas et al. 1983).

It is also reported that the inhibitory effect caused by resulting $OSCN^-$ ions is dependent on the holding temperature, pH, and microbial load. However, the LP system inhibition can be reversed by incorporating sulfhydryl-containing compounds, such as cysteine, dithiothreitol, and glutathione, as well as by high levels of catalase or by heating milk at pasteurization temperatures (Siragusa and Johnson 1989).

There are several studies performed on LP system. Fonteh et al. (2006) studied that the shelf life of raw milk could be effectively extended with small amounts of thiocyanate (20 ppm) and peroxide (20 ppm) under Cameroonian conditions by approximately 9 h without refrigeration. LP system-activated milk can be stored for as long as 21 h, allowing sufficient time for its suitable disposal. Jacob et al. (2000) observed that the exploitation of LP-thiocyanate-H_2O_2 system was an effective system against many disease causing organisms in plants and animals.

Gaya et al. (1991) found that LP system activation was shown to be a feasible procedure for controlling the development of *L.monocytogenes* in refrigerated raw milk. The bactericidal activity of LP system in raw milk prevented the growth and also reduced significantly the *L.monocytogenes* load during refrigerated storage for 5 days. According to the researchers, the LP system exhibited a bactericidal activity at 4 and 8^oC; the activity was dependent on temperature, incubation time, and strain of *L.monocytogenes*. Kamau et al.(1990) determined that the effect of the LP system on thermal resistance of bacteria suspended in milk. They reported that the thermal destruction of *L.monocytogenes* and *Staphylococcus aureus* when exposed to the LP system following the heating process was increased. By this way, LP system processes at low temperatures does not affect the nutritional value and the quality of foods. Garcia et al.(2000) investigated the combined action of the LP system and high pressure treatment on four strains of *E.coli* and *L.innocua* inoculated in milk. The aim of this research was to find a potential mild food preservation method for producing microbiologically safe foods. They concluded that the LP system immediately after high pressure treatment could be effective against *L.innocua*, but high pressure and the LP system could not exhibit a synergetic interaction on the inactivation of *E.coli*. However,

Garcia-Graells et al. (2000) found that at low cell concentration of *E.coli* used in the study were inactivated within the early hr by the help of the combined pressure-LP system treatment. They showed that the pressure resistant strain of *E.coli* needed a higher pressure to become sensitized to the LP system.

Touch et al.(2004) investigated the use of the LP system against *S.enteritidis* in tomato juice, carrot juice, milk, liquid whole egg, and chicken skin extract under various conditions. They found that the system was more effective against the organism in vegetable juices than in animal products, at low pH than at neutral pH, and at higher temperatures than at lower temperatures. They concluded that combination of the system with other preservatives or treatments would be needed to effectively inhibit growth and survival of *Salmonella* in animal products.

Björck et al. (1975) incorporated glucose and glucose oxidase into raw milk containing 0.085 mM SCN^-. They observed that *P. fluorescens* EF 1998 could not grow in the presence of 3% glucose and 0.1U of glucose oxidase, and 0.085mM SCN^- up to 10h.

Microbial growth on food surfaces is a main reason for food spoilage (Pranoto et al. 2004). Due to the fact that microbial growth in foods takes place at the surface, some methods have been developed to to reduce the growth on the surface by using antimicrobial sprays or dips (Pranoto et al. 2004).On the other hand, direct surface application of antibacterial substances has limited effect on surface flora because of the rapid diffusion of the active agent within the bulk of food or neutralization of the substances by contacting product constituents (Pranoto et al. 2004, Quattara et al. 2000).

In the indirect application, the antimicrobial agent localizes the functional efficiancy at the food surface. By this way, the agents migrate slowly to the surface and so they remain at high concentrations where they are necessary. This can be provided by edible films or coatings, which are means to carry antimicrobials (Pranoto et al. 2004). Min and Krochta (2005) conducted a study with *Penicillium commune*, which causes the deterioration of foods, as well as produce a mycotoxin. They found that lactoperoxidase system incorporated into whey protein isolate films at a level of 59 mg LP/g film inhibited growth of *P.commune*. In addition, Min et al.(2005) also demonstrated the effect of LP system on the inhibition of *L.monocytogenes* and the effect of LP system -WPI films and/or coating on smoked salmon. The WPI coatings incorporating LP system prevented the growth of *L.monocytogenes* in smoked salmon at 4 and 10^{o}C for 35 and 14d, respectively (Min et al 2005). Furthermore, they also studied

the efficiancy of WPI films with LP system on the inhibition of *Salmonella enterica* and *Escherichia coli* O157:H7. LP system -WPI films (0.15g/g) completely prevented the growth of these pathogens, (4 log CFU/cm^2) (Min et al 2005).

4.1.5.2. Lysozyme

Lysozyme (EC 3.2.1.17) is a commercially produced enzyme. It is used in food technology in order to prevent late gas blowing in cheese, and reduce sterilizing temperature for food canning (Jiang et al. 2001). In Japan, it has been used for preservation of oyster, shrimp, other seafood, sushi, sake, kimchi (pickled cabbage), Chinese noodles, potato salad, and custard (Davidson and Branen 1993).

This enzyme is a natural component of tears, plant tissues, milk, and eggs. It specifically degrades the peptidoglycan portion of the rigid bacterial cell wall (Jiang et al. 2001). It has an antimicrobial property against common food spoilage and food-borne disease-causing bacteria, including *B.cereus*, *B.stearothermophilus*, *Campylobacter jejuni*, *C.botulinum* types A,B, and E, *Clostridium butyricum*, *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium thermosaccharolyticum*, *Clostridium tyrobutyricum*, *E.coli* O157:H7, *Klebsiella pneumoniae*, *L.monocyotogenes*, *S.typhimurium*, *S.aureus*, *Vibrio cholerae*, and *Yersinia enterocolitica* (Davidson and Branen 1993). Furthermore, some strains of *C.botulinum*, *C.thermosaccharolyticum*, *C.tyrobutyricum*, *B.stearothermophilus*, and *L.monocytogenes* were effectively inhibited or lysed by lysozyme (Davidson and Branen 1993). The presence of other chemicals (e.g., EDTA), temperature, growth phase, and growth media may affect the activity of lysozyme. Kandemir et al.(2005) found that the combinational addition of lysozyme and EDTA.2H_2O_2 in pullulan film gave antimicrobial property against *Escherichia coli*. This may be used to obtain antimicrobial films to increase the safety of foods.

4.1.5.3. Glucose Oxidase

Glucose oxidase (β-D-glucose; 1-oxido-reductase) is one of the important enzymes used in the food industry. It is utilized as a stabilizing agent in wine, beer, carbonated soft drinks, mayonnaise and sauce salad dressings. In addition, the enzyme is applied in the manufacturing process of liquid egg before drying to avoid Maillard

reactions (Massa et al. 2001). Consequently, it has been used in the industry for desugaring, and as an antioxidant (Jeong et al. 1992). It is produced by molds, including *Penicillium notatum* and *Aspergillus niger* (Jeong et al. 1992).

The enzyme is an oxidoreductase. It catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide by using molecular oxygen as an electron acceptor (Jeong et al. 1992, Kana et al. 2001, Massa et al. 2001). Gluconic acid produced by this enzymatic reaction reduces the pH. It is thought that the growth of spoilage microorganisms present on food is inhibited in the more acidic environment (Jeong et al. 1992). In order to understand whether the inhibition of enterotoxic *E.coli* PM 015 and *Salmonella derby* BP 177 was caused by gluconic acid or hydrogen peroxide, Massa et al. (2001) added catalase to glucose oxidase-glucose system. Since catalase decomposes hydrogen peroxide to water and oxygen, they attributed the antibacterial effect to the result of a pH decrease by the formation of gluconic acid.

4.1.5.4. Chitinase

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of chitin to its monomer N-acetyl-D-glucosamine. Chitin is one of the major components of fungal cell wall. Therefore, the antimicrobial effect of this enzyme is against fungi. In biological research, chitinases are used to generate fungal protoplasts. The hydrolytic property of chitinases gains interest as an environmentally safe biocontrol agent. Chitinase generating organisms have been utilized to inhibit the growth of phytopathogens. *Paenibacillus* spp. is found to be a high chitinase producer (Kao et al. 2007).

4.1.6. Bioactive Proteins and Peptides

There are several bioactive proteins and peptides present in foods. These agents are also called naturally occurring antimicrobial agents.

4.1.6.1. Lactoferrin

Lactoferrin is a molecule of 80.000 D molecular weight. It is found in colostrum, milk and other exocrine fluids such as tears. It is active at neutral pH and in

the presence of bicarbonate ions (Pakkanen and Aalto 1997). It is highest in bovine colostrum. Its role in milk may also involve iron binding. It may bind the iron so tightly that it is no longer available for microbial growth (Davidson and Branen 1993). The protein has been shown to inhibit the growth of some organisms, including *E.coli*, *S.typhimurium*, *S.dysenteria*, *L.monocytogenes*, *S.mutans*, *B.stearothermophilus* and *B. subtilis* (Pakkanen and Aalto 1997). The antimicrobial activity of lactoferrin can be decreased or totally reversed in the presence of trysin, ferrous sulfate, magnessium sulfate, hematin, and citrate. It is very stable to heat (90^{o}C for 60 min) (Davidson and Branen 1993).

Lactoferricin is a peptide derived from pepsin digestion of bovine lactoferrin. It has a low-molecular-weight, single-chain peptide of 25 amino acid residues (Davidson and Branen 1993). It also has antimicrobial activities against various bacteria and *Candida albicans* (Pakkanen and Aalto 1997).

4.1.6.2. Phosvitin

Phosvitin is an egg yolk protein. It has a molecular weight between 36.000 and 40.000 D (Lee et al. 2002). This egg yolk phosphoglycoprotein is a highly phosphorylated serine-rich glycoprotein. It contains approximately 10 % phosphorus and 6.5 % carbohydrates (Khan et al. 1998).

Phosvitin has an antimicrobial spectrum because it is an amphiphilic protein with chelating ability. The protein is a good candidate for damaging the outer membrane of Gram-negative bacteria. It binds and limits iron which is necessary for bacterial growth (Khan et al. 2000). Kahan et al.(2000) demonstrated that phosvitin damaged the outer membrane and killed *E.coli* under thermal stress. In addition, to suppress the lipid oxidation reactions in food is of great importance to the food industry. The aim is to prevent undesirable changes to food products that result in loss of nutritional value, formation of off-flavors, and production of potential toxins. Phosvitin also has an antioxidant potential (Nakamura et al. 1998). Lee et al. (2002) investigated the antioxidant property of phosvitin in muscle food. The researchers suggested that due to its insufficient antioxidant activity, it should be utilized together with other antioxidants.

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

Whole milk used to produce LP enzyme was obtained from a local farm in İzmir in every two months (2005-2006). Potassium thiocyanate was purchased from Sigma-Aldrich (Stenheim, Germany). Toyopearl sulphopropyl (SP) cation-exchanger (SP-550C, fast flow, size: 100 μm) was purchased from Supelco (Bellefonte, PA, USA). Dialysis tubes (cut off: 12000 MW), dextran (from *Leuconostoc mesenteroides*, 73.200 MW), ABTS (2,2-Azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)), and the sodium salt of alginic acid (from *Macrocystis pyrifera*, viscosity of 2% solution at 25 °C is 3500 cpc) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Rennet was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA). For microbiological analyses, nutrient agar (NA), DeMan, Rogosa and Sharpe (MRS) agar, plate count agar (PCA), violet red bile agar (VRBA), peptone water, and tween 80 were purchased from Fluka (Spain), nutrient broth (NB) and MRS broth were obtained from Merck (Germany).

5.2. Methods

5.2.1. Alginate Films Incorporating Lactoperoxidase

5.2.1.1. Preparation of Lactoperoxidase from Bovine Milk

The LP was produced from bovine whey by using the method of Mecitoğlu and Yemenicioğlu (2007). Skim milk was prepared by centrifugation at 5000*g* for 20 min at 30 °C. It was filtered through a cheese-cloth, and then warmed to 37 °C. After the temperature reached to 37 °C, 90 mg rennet was added to 900 mL of skim milk and kept at 37 °C for 1 hr. The rennet whey was separated by filtration through a cheese-cloth

and centrifuged at 10000*g* for 25 min at 4 °C for removing the remaining fat and insoluble proteins.

Chromatography was carried out using cation exchange chromatography coloumn, a 11.5 cm x 2.8 cm Toyopearl SP column previously equilibrated with 0.05 M Na-phosphate buffer at pH 6.5. Rennet whey (400 mL) was passed through the column and then washed with 500 mL of 0.05 M sodium phosphate buffer (pH 6.5). Adsorbed proteins were eluted with a linear concentration gradient of 0 – 0.55 M NaCl in 300 mL of 0.05 M sodium phosphate buffer (pH 6.5). Elute fractions of 10 mL were collected in tubes. The absorbance of each tube was measured at 280 nm and LP activity was determined. LP activity test was performed based on a color change. The aliquot (0.2 mL) from each tube was reacted with 1 mL of 0.65 mM ABTS and 0.5 mL of 0.1 mM H_2O_2. The tubes having dark or close to dark blue colors were collected. Finally, the solution was dialysed using distilled water for 24 hr at 4 °C. After dialysis, 250-300 mg of dextran as a supporting agent was mixed with the dialyzed enzyme and it was lyophilized in a Labconco freeze-dryer (FreeZone 61, Kansas City, MO, USA), working approximately at -47 °C collector temperature and 50-100 x 10^{-3} mBar vacuum. The lyophilized enzyme was stored at -18 °C prior to analyses.

5.2.1.2. Determination of Lyophilized Lactoperoxidase Activity

LP activity was spectrophotometrically determined in triplicate by monitoring the oxidation of ABTS using a (Shimadzu, model 2450, Tokyo, Japan) spectrophotometer together with a constant temperature cell holder running at 30 °C (Chávarri et al. 1998). Briefly, 0.1 mL of enzyme solution was mixed with 2.3 mL, 0.65 mM ABTS (prepared in Na-phosphate buffer) in a cuvette. The reaction was initiated by adding 0.1 mL H_2O_2 (0.2 mM) to the mixture. The reaction was monitored for 5 min at 412 nm. The enzyme activity was estimated from the slope of the initial linear portion of absorbance vs. time curve. Finally, the results were expressed as Unit (0.001 absorbance change in 1 min).

5.2.1.3. Preparation of Alginate Films

The alginate films were produced as described in Mecitoğlu and Yemenicioğlu (2007). To prepare films, lyophilized LP preparation (0.26-0.597 mg) was dissolved per gram of 2% (w/v) alginic acid solution by mixing slowly with a magnetic stirrer. 10 g portions of this solution were then spread onto the glass Petri dishes (9.5 cm in diameter). The Petri dishes were dried at room temperature for three days and 0.8 mL of 0.3 M $CaCl_2$ was pipetted onto the films for cross-linking, but before using the films they were washed with 10 mL sterile deionized water for 15 sec in order to remove excessive $CaCl_2$.

5.2.1.4. Determination of Lactoperoxidase Activity Released from the Alginate Film

The release tests were performed in a refrigerated incubator at 4 °C. The alginate film incorporated with 900 U/cm^2 LP was placed in a glass Petri dish containing 50 mL distilled water (4 °C). The Petri dish was then covered with a parafilm in order to prevent moisture loss and was incubated at 4 °C for 24 hr with stirring at 200 rpm using a magnetic stirrer (2 cm long Teflon coated rod). The LP activity in release test solution was monitored by taking 0.2 mL aliquots in triplicate from the release test solution at different time intervals. The LP activity measurements were conducted by mixing 0.2 mL of the aliquot with 2.2 mL of 0.65 mM ABTS prepared in 0.1 M Na-phosphate buffer at pH 6.0 and 0.1 mL of 0.4 mM H_2O_2. The absorbance of the reaction mixtures was measured at 412 nm using a spectrophotometer (Shimadzu, model 2450, Tokyo, Japan).

5.2.1.5. Determination of Immobilized Lactoperoxidase Activity in the Alginate Film

The immobilized LP activity in the alginate film was determined by slightly modifying the method of Mecitoğlu and Yemenicioğlu (2007). The dried and cross-linked alginate film incorporating 900 U/cm^2 LP was halved with a clean razor and one of the halves was placed into a glass Petri dish containing 23 mL of 0.65 mM ABTS

solution (prepared in 0.1M Na-phosphate buffer at pH 6.0) and 2 mL of 200, 400, or 800 μM H_2O_2 solution which was brought to 30 °C prior to mixing. The Petri dishes were incubated at 30 °C and stirred at 200 rpm with a magnetic stirrer. The LP activity was monitored by measuring the reaction mixture absorbance at 412 nm at different time intervals and it was determined from the slope of the initial linear portion of absorbance vs. time curve. The measurements were repeated for the remaining halves of the film and average of two measurements was considered in all measurements. The activity was expressed as U (0.001 absorbance change in one minute) per cm^2 of the film.

5.2.1.6. Antimicrobial Activity of Alginate Films

5.2.1.6.1. Bacterial Strains

The bacterial strains used in this study were *Listeria innocua* (NRRL-33314), *Escherichia coli* (NRRL-B-3008), *Pseudomonas fluorescens* (NRRL B-253). These strains were supplied from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois. The frozen stock cultures were maintained in nutrient broth containing 15% glycerol at - 80 °C prior to analyses.

5.2.1.6.2. Determination of Antimicrobial Activity of Alginate Films Incorporating Lactoperoxidase

Alginate films with or without LP were prepared as it was described in section 5.2.1.3. The films were washed with 25 mL sterile deionized water for 15 sec and the discs (1.3 cm in diameter) were cut aseptically using a cork borer. The discs having 900 U/cm^2 LP were placed into the test tubes containing 3 mL sterile Nutrient broth, 0.5 mL culture, 0.1 mL potassium thiocyanate (final concentration in reaction mixture: 4000 μM), and 0.1 mL H_2O_2 (final concentration in tubes: 200, 400, or 800 μM). *E. coli*, *L. innocua* or *P. fluorescens* were used as test cultures in the reaction mixtures. *E. coli* and *L. innocua* were incubated using nutrient broth at 37 °C for 16-18 hr and *P. fluorescens* was incubated using nutrient broth at 26°C for 16-18 hr.

In order to determine the effectiveness of LP system at different concentrations of H_2O_2 and constant thiocyanate concentration, six different reaction mixtures were prepared. Reaction mixture #1 was the control sample having only the nutrient broth (NB), sterile water, and culture; #2 contained NB, sterile water, culture, and disc; #3 contained NB, sterile water, culture, disc, and thiocyanate; #4 contained NB, culture, disc, thiocyanate, and 200 μM H_2O_2; #5 contained the same constituents as in #4 except the concentration of H_2O_2 which was 400 μM instead of 200 μM; #6 was the same as #4 except the concentration of H_2O_2 was 800 μM instead of 200 μM. The test tubes having *E. coli* and *L. innocua* were incubated at 37 °C for 24 hr and the tubes inoculated with *P. fluorescens* were incubated at 26 °C for 24 hr. *E. coli*, *L. innocua* or *P. fluorescens* were enumerated on nutrient agar by taking samples from reaction mixtures at 0, 6 and 24 hr of incubation. Pour plate method was performed and the duplicate plates for each dilution were incubated at 37°C/24 hr for *E. coli*, at 37 °C/48 hr for *L. innocua*, and at 26 °C/48 hr for *P. fluorescens*. The microbial counts were expressed as $\log_{10}$ cfu/mL. The residual H_2O_2 in the reaction mixtures were determined using semi-quantitative H_2O_2 test papers (detection limit 1 mg/ L, Quantofix Peroxide 100, Macherey-Nagel, Düren, Germany) at 0, 6 and 24 hr of incubation.

To determine the effectiveness of LP system at different concentrations of thiocyanate (1000 μM or 2000 μM in the reaction mixture) and constant H_2O_2 concentration (200 μM), six different reaction mixtures were prepared. Reaction mixture #1 was the control sample containing only NB, sterile water, and culture; #2 contained NB, sterile water, culture, and disc; #3 had NB, sterile water, culture, disc, and 1000 μM thiocyanate; #4 contained NB, culture, disc, 1000 μM thiocyanate and 200 μM H_2O_2; #5 contained NB, sterile water, culture, disc, and 2000 μM thiocyanate; #6 contained broth, culture, disc, 2000 μM thiocyanate and 200 μM H_2O_2.

The test tubes having *E. coli* and *L. innocua* were incubated at 37 °C for 24 hr and the tubes inoculated with *P. fluorescens* were incubated at 26 °C for 24 hr. *E. coli*, *L. innocua* or *P. fluorescens* were enumerated on nutrient agar by taking samples from reaction mixtures at 0, 6 and 24 hr of incubation. Pour plate method was performed and the duplicate plates for each dilution were incubated at 37 °C/24 hr for *E. coli*, at 37 °C/48 hr for *L. innocua*, and at 26 °C/48 hr for *P. fluorescens*. The microbial counts were expressed as $\log_{10}$ cfu/mL. The residual H_2O_2 in the reaction mixtures were

determined using semi-quantitative H_2O_2 test papers (detection limit 1 mg/L, Quantofix Peroxide 100, Macherey-Nagel, Düren, Germany) at 0, 6 and 24 hr of incubation. All the experiments were performed in duplicate using LP produced from different batches of milk samples.

Data was analyzed by one-way analysis of variance (ANOVA) using Minitab version 13 (Minitab, Inc., State College, PA, U.S.A.). Means with a significant difference ($p<0.05$) were compared using the Tukey's test.

5.2.1.7. Application of Lactoperoxidase Incorporated Alginate Films to Calamari

Fresh and cleaned calamari (5 kg) was purchased from a local seafood market in İzmir. It was transferred to the laboratory in ice in the same day. In the laboratory, calamari was washed in tab water and cut into rings. The calamari rings were randomly separated into four batches. The treatments applied to each batch were as follows:

1) Untreated samples were placed in a flask containing 45 mL sterile deionized water;

2) Samples coated with alginate film solution without having lactoperoxidase, cross-linked with 0.3 M $CaCl_2$, and placed in flasks containing 45 mL sterile deionized water;

3) Samples coated with alginate film solution incorporating lactoperoxidase (22 mg/10 g film solution) and 4000 µM KSCN, cross-linked with 0.3 M $CaCl_2$, and placed in flasks containing 45 mL 4000 µM H_2O_2;

4) Samples coated with alginate film solution incorporating lactoperoxidase (22 mg/10 g film solution) and 4000 µM KSCN, cross-linked with 0.3 M $CaCl_2$, and placed in flasks containing 45 mL 8000 µM H_2O_2.

The untreated and treated calamari samples were stored at 4 °C for 7 days. Total viable count was performed at days 0, 1, 2, 3, 5, and 7. The calamari samples (10 g) were transferred aseptically into stomacher bags containing 90 mL of sterile peptone water (0.1%) and homogenized in a stomacher (Interscience, France) at medium speed for 60 sec. For each sample, appropriate serial decimal dilutions were prepared using sterile peptone water (0.1%) and plated in duplicate on plate count agar. The plates were

incubated at 30 °C for 48 hr and microbial counts were determined. The counts were expressed as $\log_{10}$ cfu/g.

The experiment was carried out in triplicate. Data was analyzed by one-way analysis of variance (ANOVA) using Minitab version 13 (Minitab, Inc., State College, PA, U.S.A.). Means with a significant difference ($p<0.05$) were compared using the Tukey's test.

5.2.2. Alginate Films Incorporating Lactic Acid Bacteria

5.2.2.1. Bacterial Strains

Lactobacillus delbrueckii subsp. *lactis* (NRRL B-4525), *Lactobacillus casei* (NRRL B-441) were obtained from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois. The frozen stock cultures of *Lactobacillus casei* (NRRL B-441), *Lactobacillus delbrueckii* subsp. *lactis* (NRRL B-4525) and *Lactobacillus plantarum* (DSM NR 1954) were maintained in MRS broth containing 15% glycerol at -80 ℃ prior to the analysis.

5.2.2.2. Test of the Ability of Lactic Acid Bacteria to Produce Hydrogen Peroxide

Two loops from the frozen stock cultures of *L. casei* and *L . delbrueckii* subsp. *lactis* were inoculated with 25 mL MRS broth and incubated anaerobically (in an incubator 5% CO_2 and 50% humidity) at 37 °C for 24 hr. Then, the culture broth (1 mL) was again inoculated with 90 mL MRS broth and incubated anaerobically at 37 °C for 24 hr. During the second incubation, samples were taken for bacterial enumeration at 0, 4, 6, 9, and 24 hr. They were serially diluted in 0.1% peptone water and pour plate method was applied using MRS agar. The plates were incubated at 37^{o}C for 48 hr. At 0, 4, 6, 9, and 24 hr samples were tested for optical density (OD) and H_2O_2 concentration. OD measurements were done at 650 nm using a spectrophotometer (Shimadzu, model 2450, Tokyo, Japan), and H_2O_2 concentration (mg/L) was determined by using semi-quantitative test papers (detection limit 1 mg/L, Quantofix Peroxide 100, Macherey-

Nagel, Düren, Germany). The concentrations of H_2O_2 produced during the growth of these two lactic acid bacteria were determined.

5.2.2.3. Test for the Ability of *L. delbrueckii* subsp. *lactis* and *L. plantarum* to Produce H_2O_2 and Lactic Acid at Different Storage Temperatures

Two loops from the frozen stock cultures of *L. delbrueckii* subsp. *lactis* and *L. plantarum* were inoculated into 25 mL MRS broth and incubated anaerobically at 37 °C (in the CO_2 incubator) for 16 hr. Then, the culture broth (1 mL) was aseptically inoculated into 99 mL MRS broth and incubated at 4 and 23 °C for 144 hr (7 days). The pH values, lactic acid bacteria count, H_2O_2 concentration, and lactic acid content (D and L form respectively) were determined at predetermined time intervals of incubation. The pH was monitored using a pH-meter (Microprocessor pH meter, Hanna Instruments, Romania). Lactic acid bacteria counts were enumerated using MRS agar by pour plate method. The duplicated plates were incubated anaerobically at 37 °C for 48 hr. The H_2O_2 concentration was determined using semi-quantitative test papers (detection limit 1 mg/ L, Quantofix Peroxide 100, Macherey-Nagel, Düren, Germany) and the lactic acid content was determined by test kits (Boehringer Mannheim, Germany).

5.2.2.4. Preparation of Lyophilized Culture

MRS broth (10 mL) was inoculated with a loop of frozen stock cultures of *L. delbrueckii subsp. lactis*, and *L. plantarum*. The broth was incubated anaerobically at 37 °C for 24 hr (in an incubator 5% CO_2 and 50% humidity). Then, 10 mL incubated broth was inoculated with 240 mL sterile MRS broth and incubated anaerobically at 37 °C for 16 hr (in an incubator 5% CO_2 and 50% humidity). The culture was washed in sterile saline solution (0.9 % NaCl w/v) after two consecutive centrifugations (5000*g*) at 4 °C for 15 min. The pellets were suspended in reconstituted skim milk containing 7% (w/v) sucrose, and the solution was stored at -20 °C before lyophilization. The frozen solution was lyophilized in a Labconco freeze-dryer (FreeZone 61, Kansas City, Mo,

USA), working approximately at - 47 °C collector temperature and 50-100 x 10^{-3} mBar vacuum.

5.2.2.5. Preparation of Alginate Films Incorporating Lactic Acid Bacteria

To prepare films, 2-12 mg of lyophilized lactic acid bacteria (*L. delbrueckii subsp. lactis*, and *L. plantarum*) was dissolved in 2% (w/v) alginic acid solution by mixing slowly with a magnetic stirrer. 10 g portions of this solution were then spread onto glass Petri dishes (9.5 cm in diameter). The Petri dishes were dried at room temperature for 1 hr and 5 mL, 0.3 M $CaCl_2$ was pipetted into them to cross-link the dried films.

5.2.2.5.1. Determination of the Number of Free and Immobilized Lactic Acid Bacteria in Alginate Films

The free lactic acid bacteria in the alginate films were determined by using a shaking incubator (Barnstead International, LabLine 4000-BCE, Iowa, USA) at 25 °C. The cross-linked film was weighed and then placed in a Petri dish containing 25 mL, 0.1% steril peptone water. The Petri dish was incubated for 5 min with continuous stirring at 90 rpm. The amounts of lactic acid bacteria were determined by taking 1mL aliquots from the solution in the Petri dish and serially diluted with 0.1 % peptone water. Pour plate method using MRS agar was employed by using duplicate plates. The plates were incubated anaerobically at 37 °C (5 % CO_2, 50 % humidity) for 48 hr and counts were expressed as $\log_{10}$ cfu/g film.

After 5 min of incubation, the alginate film incorporated with lactic acid bacteria was homogenized using a homogenizator (Waring Commercial Blendor, New Hartford, CT, 06057, USA) to determine the immobilized lactic acid bacteria in the film. The serial dilutions were made using 0.1% peptone water and MRS agar was used to enumerate lactic acid bacteria. The Petri dishes were incubated at 37 °C (5 % CO_2, 50 % humidity) for 48 hr. The counts were expressed as $\log_{10}$ cfu/g film.

5.2.2.5.2. Determination of the Stability of the Lactic Acid Bacteria Incorporated into the Alginate Film Forming Solution

To prepare films, 60 mg of lyophilized lactic acid bacteria (*L. delbrueckii subsp. lactis*, and *L. plantarum*) was added in 2 % alginic acid solution. The film solutions were stored at 4 °C for 7 days in firmly-sealed Petri dishes. At different time periods, the film forming solutions were taken and cross-linked by pipetting 5 mL 0.3 M $CaCl_2$ into the Petri dishes. The number of lactic acid bacteria in the film was enumerated at days 0, 1, 3, and 7. Then, the free and immobilized lactic acid bacteria counts were determined to evaluate the stability of lactic acid bacteria. The experiments were performed as stated in section 5.2.2.5.1.

5.2.2.5.3. Determination of the Stability of the Lactic Acid Bacteria in Powdered Alginic Acid

The alginic acid was mixed with 60 and 120 mg lactic acid bacteria (*L. delbrueckii subsp. lactis*, and *L. plantarum*) and stored at 4°C. To determine the lactic acid bacteria counts, 50 mg alginic acid containing lactic acid bacteria was taken and dissolved in 10 mL sterile peptone water (0.1 %). The number of lactic acid bacteria was determined by serially-diluting using sterile peptone water and pour plating with MRS agar. The Petri dishes were incubated anaerobically at 37 °C (5 % CO_2, 50 % humidity) for 48 hr. The results were expressed as $\log_{10}$ cfu/g.

5.2.3.4. Applications of Alginate Films Incorporating Lactic Acid Bacteria to Fresh Beef Cubes

The vacuum packaged beef loaf (approximately 4.5 kg) was obtained from Pınar Et A.Ş. (İzmir, Turkey). The outer surfaces of the meat were removed to avoid possible contamination before cutting into cubes. The beef cubes were separated into four batches and treated as follows: 1) untreated; 2) treated with alginate film solution and cross-linked with 0.3 M $CaCl_2$; 3) treated with 90 mg *L. debrueckii* subsp. *lactis* per 10 g of alginate solution and cross-linked with 0.3 M $CaCl_2$; 4) treated with 90 mg *L. plantarum* per 10 g of alginate solution and cross-linked with 0.3 M $CaCl_2$. The cubes

first coated with the alginate film solution and then immersed into 0.3 M $CaCl_2$ solution for 2-3 sec to cross-link. The uncoated and coated samples were placed in sterile Petri dishes, tightly wrapped with a stretch film and stored at 4^{o}C for 14 days. At days 0, 1, 2, 3, 7, and 14, approximately 10 g samples were taken from each treatment, and they were homogenized using a stomacher (Interscience, France). Each homogenized sample was serially diluted in 0.1% sterile peptone water and plated in duplicate on MRS agar. The plates were incubated anaerobically at 37 °C (5 % CO_2, 50 % humidity) for 48 hr and lactic acid bacteria counts were determined. The counts were expressed as $\log_{10}$ cfu/g.

CHAPTER 6

RESULTS AND DISCUSSIONS

In the present study, the lyophilized lactoperoxidase and lactic acid bacteria were incorporated into the edible alginate films. Lactoperoxidase (LP) forms the naturally occurring antimicrobial system milk. Thus, one of the aims of this study is the exploitation of the naturally occurring antimicrobial mechanism by adapting it in alginate films used frequently for coating of meat, poultry and fish (Lindstrom et al., 1992). The lactic acid bacteria also exist naturally in meat, poultry and fish. In fact, enrichment of food by lactic acid bacteria is currently applied extensively to create an additional safeguard for temperature abuse conditions occurred during storage of refrigerated meat and meat products. The rapid development of lactic acid bacteria during temperature abuse conditions prevents growth of pathogenic bacteria and food is spoiled by nontoxic lactic acid bacteria by causing easily detectable acidity. This prevents poisoning by pathogens which mostly cause no detectable change in food. The second aim of this work is to incorporate lactic acid bacteria into alginate films. By this way, the application of bacterial cultures to meat and meat products will become a very practical process which needs no dealing with liquid culture and complicated aseptic spraying equipment.

6.1. Incorporation of Lactoproxidase into Alginate Films

6.1.1. Determination of the LP Activity Released from the Alginate Films

Release tests were performed at 4 °C in distilled water (50 mL, stirring rate 200 rpm) for 24 hr. No LP activity release was determined from the alginate films incorporated with 900U/cm^2 LP. The alginate films are consisted of linear copolymers of D-mannuronic acid and L-guluronic acid which are cross-linked by the $CaCl_2$. The enzyme has a high isoelectric point (pI 9.6). Thus, it may bind to films by the negatively charged carboxylic acid groups on polymeric chains of alginate. In this research, the

enzyme was prepared with dextran. Thus, the H-bonding of dextran to enzyme and alginate could also make a contribution to the immobilization of the enzyme (Mecitoğlu and Yemenicioğlu 2007).

6.1.2. Determination of the LP Activity Immobilized in the Alginate Films

The extent to which lactoperoxidase activity was affected while changing the concentration of H_2O_2 in the reaction mixture was investigated. Mecitoğlu and Yemenicioğlu (2007) found that between 2 and 24 µM H_2O_2 concentrations, a two-fold increase in H_2O_2 concentration led to 1.5-2.5-fold increase in lactoperoxidase activity of films incorporated with 1200 U/cm^2 LP. In this study, 200, 400, and 800 µM H_2O_2 were used to examine the LP activity of films incorporated with 900 U/cm^2 LP. The results revealed that the lowest H_2O_2 concentration (200 µM) used in the study gave the highest enzyme activity. The increase in H_2O_2 concentration (400 µM) declined the activity, but when 800 µM H_2O_2 was used, it was slightly increased again. This work approved the results of Fonteh et al. (2005) that above 200 µM H_2O_2 concentration LP inhibited possibly due to suicidal inhibition. This indicated the complexity of the LP system.

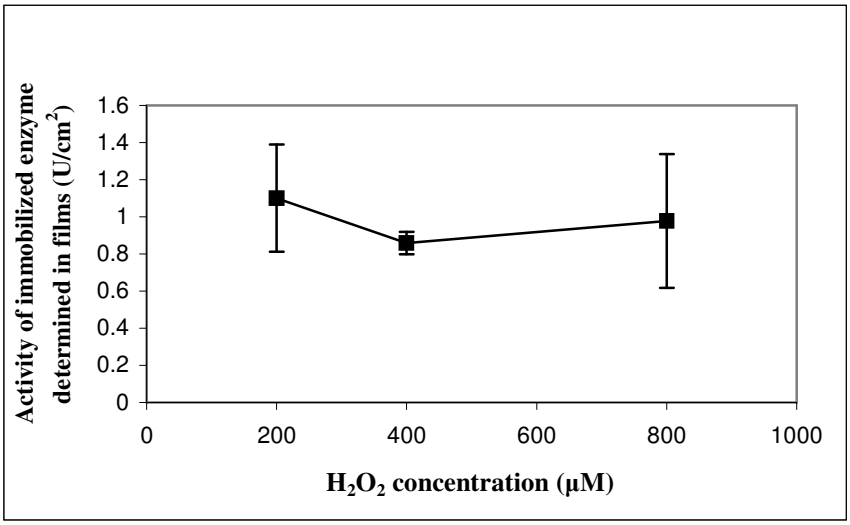


Figure 6.1. The immobilized lactoperoxidase activity in different H_2O_2 concentrations.

6.1.3. Antimicrobial Activity of LP-H_2O_2-Thiocyanate System

The antimicrobial activity of LP-H_2O_2-thiocyanete system on *Escherichia coli* (NRRL B-3008), *Listeria innocua* (NRRL B-33314), and *Pseudomonas fluorescens* (NRRL B-253) was investigated by using 1.3 cm in diameter discs prepared from alginate films incorporated with 900 U/cm^2 LP in the presence of 0, 200, 400, or 800 μM H_2O_2 and 4000 μM KSCN in one set of experiments and in the other set of experiments the antimicrobial activity of LP system on the previously mentioned microorgansims by using the alginate discs incorporating 900 U/cm^2 LP in the presence of 0 or 200 μM H_2O_2 and 0, 1000, and 2000 μM KSCN .

6.1.3.1. Effects of LP System in Reaction Mixtures Containing Alginate Films Incorporating LP against *E.coli*

The results of the effect of LP system against the growth of *E. coli* were summarized in Table 6.1. According to these results, the LP system had an inhibitory effect on the growth of *E. coli* in the presence of 200, 400, and 800 μM H_2O_2. Incubation time had a significat effect on each treatment ($p<0.05$). The growth of the microorganism was delayed up to the 6th hr (Figure 6.2). It was observed that the bacteria were inhibited in the presence of high H_2O_2 especially in the 2nd trial (Figure 6.2). Thus, findings showed that the enzymes used in the 1st and 2nd trials had different kinetic properties. The different H_2O_2 consumption rates also supported this hypothesis. Although the enzyme activity used in the films was at the same level (900 U/cm^2), the H_2O_2 was used more rapidly in the 2nd trial than in the 1st one (Table 6.3). On the other hand, the bacteria began to grow immediately between 6 and 24 hr at the same levels as the control regardless of the treatments with the system. It could be explained by the absence of H_2O_2 to be used for the inhibitory reaction. However, these films could be applied for minimally-processed foods stored at 0-4 °C. Under these conditions, the enzyme would work more slowly than at 37 °C, and therefore, the antimicrobial effectiveness would be prolonged.

Table 6.1. *E. coli* counts in reaction mixtures having different concentrations of H_2O_2 during 24 hr incubation at 37 °C.

	No	Total LP activity in discs (U/cm^2)[1]	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	*E. coli* counts (log_{10} cfu/mL)		
					Incubation time at 37 °C (hr)		
				0	6	24	
1st Trial	1[2]	-	-	-	3.8[c]	7.5[b]	9.3[a]
	2[3]	-	-	-	4.3[c]	7.8[b]	9.0[a]
	3	900 (500)[4]	4000	-	5.7[c]	8.0[b]	9.0[a]
	4	900 (500)	4000	200	5.7[b]	5.8[b]	8.8[a]
	5	900 (500)	4000	400	5.0[c]	5.8[b]	9.2[a]
	6	900 (500)	4000	800	5.6[c]	6.2[b]	8.8[a,]
2nd Trial	1[2]	-	-	-	4.2[c]	8.7[b]	9.2[a]
	2[3]	-	-	-	4.2[c]	8.7[b]	9.2[a]
	3	900 (597)	4000	-	4.3[c]	8.6[b]	9.3[a]
	4	900 (597)	4000	200	4.3[c]	6.7[b]	9.1[a]
	5	900 (597)	4000	400	4.2[b]	3.0[c]	8.8[a]
	6	900 (597)	4000	800	4.2[b]	2.0[c]	8.6[a]

[1] LP activity of films used for 1st trial: 1188 U/disc (660 µg/disc) and for 2nd trial: 1188 U/disc (788 µg/disc)
[2] Reaction mixture contains only nutrient broth and *E. coli*
[3] Reaction mixture contains nutrient broth, *E. coli* and discs without lactoperoxidase enzyme
[4] Lactoperoxidase enzyme content as µg per disc
[a-c] Row means having a different letter are significantly different ($P<0.05$).

Table 6.2. The change of H_2O_2 concentration in reaction mixtures during 24 hr incubation at 37 °C.

	No	Initial H_2O_2 concentration (µM)	H_2O_2 concentration (µM)		
			Incubation time at 37 °C (hr)		
			0	6	24
1st Trial	1	200	88-200	-	-
	2	400	294	0-29	-
	3	800	294-800	29-88	-
2nd Trial	1	200	88-200	-	-
	2	400	88-294	-	-
	3	800	294-800	29-88	-

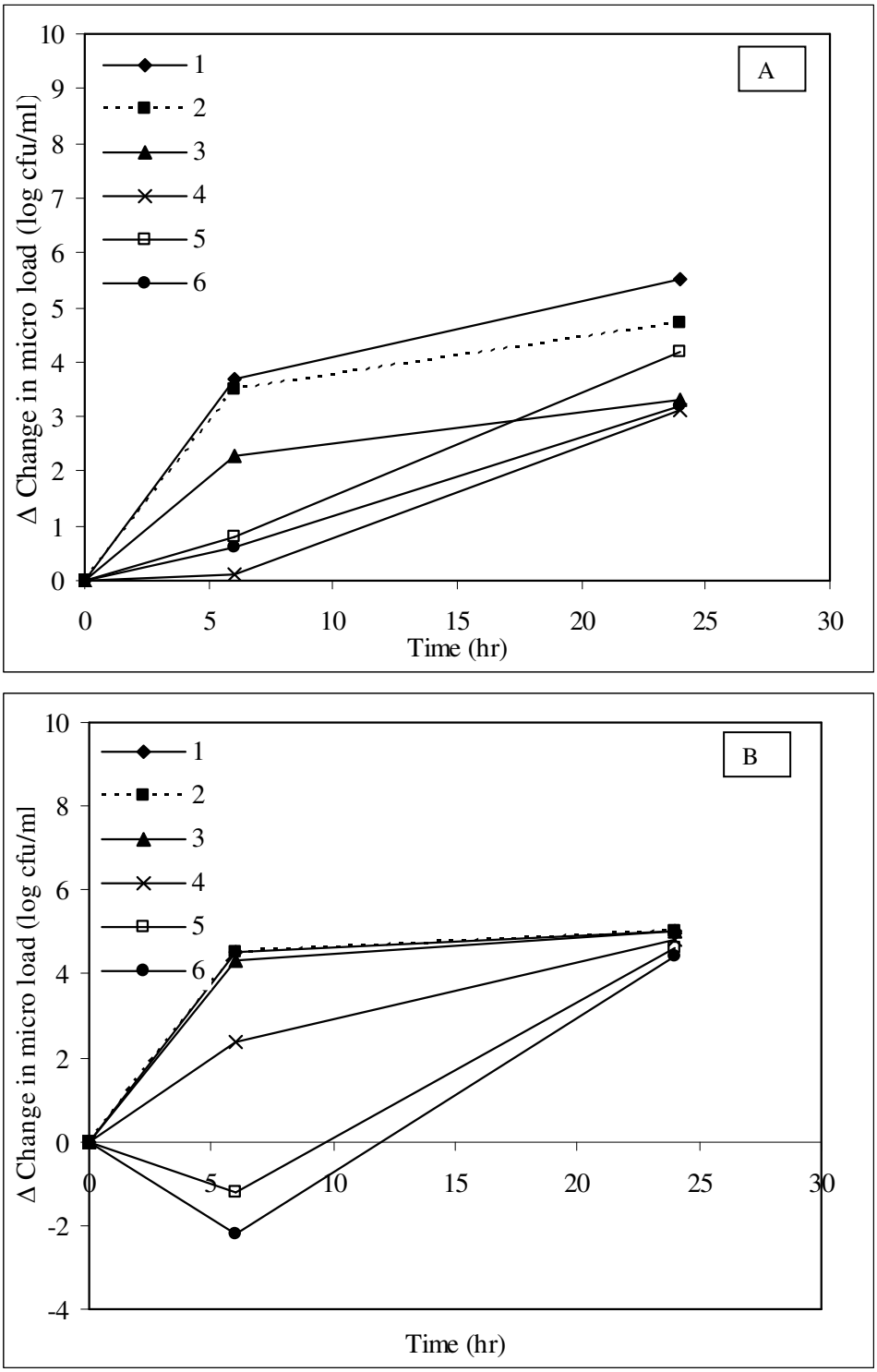


Figure 6.2. Change of microbial counts (log_{10} cfu/mL) of *E. coli* in reaction mixtures containing 4000 μM KSCN, 1188 U lactoperoxidase/disc (this activity was reached by using 660 μg/disc (A: 1st trial) or 788 μg/disc (B: 2nd trial) enzyme for different LP preparations) and different concentrations of H_2O_2. Composition of reaction mixtures; 1: Nutrient broth (NB)+*E. Coli* (E) + sterile deionized water (W); 2: NB+E+Alginate disc (A)+W; 3: NB+E+Lactoperoxidase incorporated alginate disc (L) + Potassium thiocyanate (T)+W; 4: NB+E+L+T+H_1 (200 μM); 5: NB+E+L+T+H_1 (400 μM); 6: NB+E+L+T+H_1 (800 μM).

The effect of LP system in presence of different concentrations of KSCN (1000 or 2000 μM) and H_2O_2 (0, 200 μM) against *E. coli* was also investigated. The results were given in Table 6.3. In this case, bacteriostatic effect, instead of inhibitory action, occurred when 1000 or 2000 μM KSCN, and 200 μM H_2O_2 were used. The obtained antimicrobial effect was also lasted up to 6 hr. Between 6 and 24 hr, the microorganism started to develop readily. The present work showed that the antimicrobial effect did not improve when the thiocyanate concentration was increased from 1000 to 2000 μM in the presence of 200 μM H_2O_2. In fact, the increase in KSCN concentration caused a decline in the effectiveness of LP system (Figure 6.3.). As seen from Table 6.4, the H_2O_2 concentration was declined to an undetectable level within the 6^{th} hr. Thus, it is hard to compare the H_2O_2 consumption rates of different reaction mixtures. However, higher antimicrobial activity of 1000 μM KSCN than 2000 μM KSCN in presence of 200 μM H_2O_2 suggested that the limiting factor for the effectiveness of LP system is H_2O_2. This was expected since the short lived antimicrobial products of KSCN oxidation should have been formed and then degraded more rapidly in presence of higher concentraiton of KSCN.

Table 6.3. *E. coli* counts in reaction mixtures having different concentrations of KSCN during 24 hr incubation at 37 °C.

	No	Total LP activity in discs (U/cm^2)[1]	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	*E. coli* counts (log_{10} cfu/mL)		
					Incubation time at 37 °C (hr)		
					0	6	24
1st Trial	1[2]	-	-	-	3.0[c]	7.4[b]	9.2[a]
	2[3]	-	-	-	3.0[c]	7.5[b]	9.3[a]
	3	900 (344)[4]	1000	-	4.1[c]	7.8[b]	9.2[a]
	4	900 (344)	1000	200	4.9[c]	6.3[b]	9.1[a]
	5	900 (344)	2000	-	4.3[c]	7.8[b]	9.1[a]
	6	900 (344)	2000	200	4.3[c]	6.2[b]	9.1[a]
2nd Trial	1[2]	-	-	-	3.2[c]	8.1[b]	9.2[a]
	2[3]	-	-	-	3.2[c]	8.3[b]	9.3[a]
	3	900 (597)[4]	1000	-	3.4[c]	8.2[b]	9.2[a]
	4	900 (597)	1000	200	3.3[b]	3.5[b]	9.0[a]
	5	900 (597)	2000	-	3.5[c]	8.4[b]	9.3[a]
	6	900 (597)	2000	200	3.2[c]	5.8[b]	9.2[a]

[1] LPS activity of films used for 1st trial: 1188 U/disc (454 µg/disc) and for 2nd trial: 1188 U/disc (788 µg/disc)
[2] Solution contains only nutrient broth and *E. coli*
[3] Solution contains nutrient broth, *E. coli* and discs without lactoperoxidase enzyme
[4] Lactoperoxidase enzyme amount in µg in each disc
[a-c] Row means having a different letter are significantly different ($P<0.05$).

Table 6.4. The change of H_2O_2 concentration in reaction mixtures during 24 hr incubation at 37 °C.

	No	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	H_2O_2 concentration (µM)		
				Incubation time at 37 °C (hr)		
				0	6	24
1st Trial	1	1000	200	88-200	-	-
	2	2000	200	88-200	-	-
2nd Trial	1	1000	200	88-200	-	-
	2	2000	200	88-200	-	-

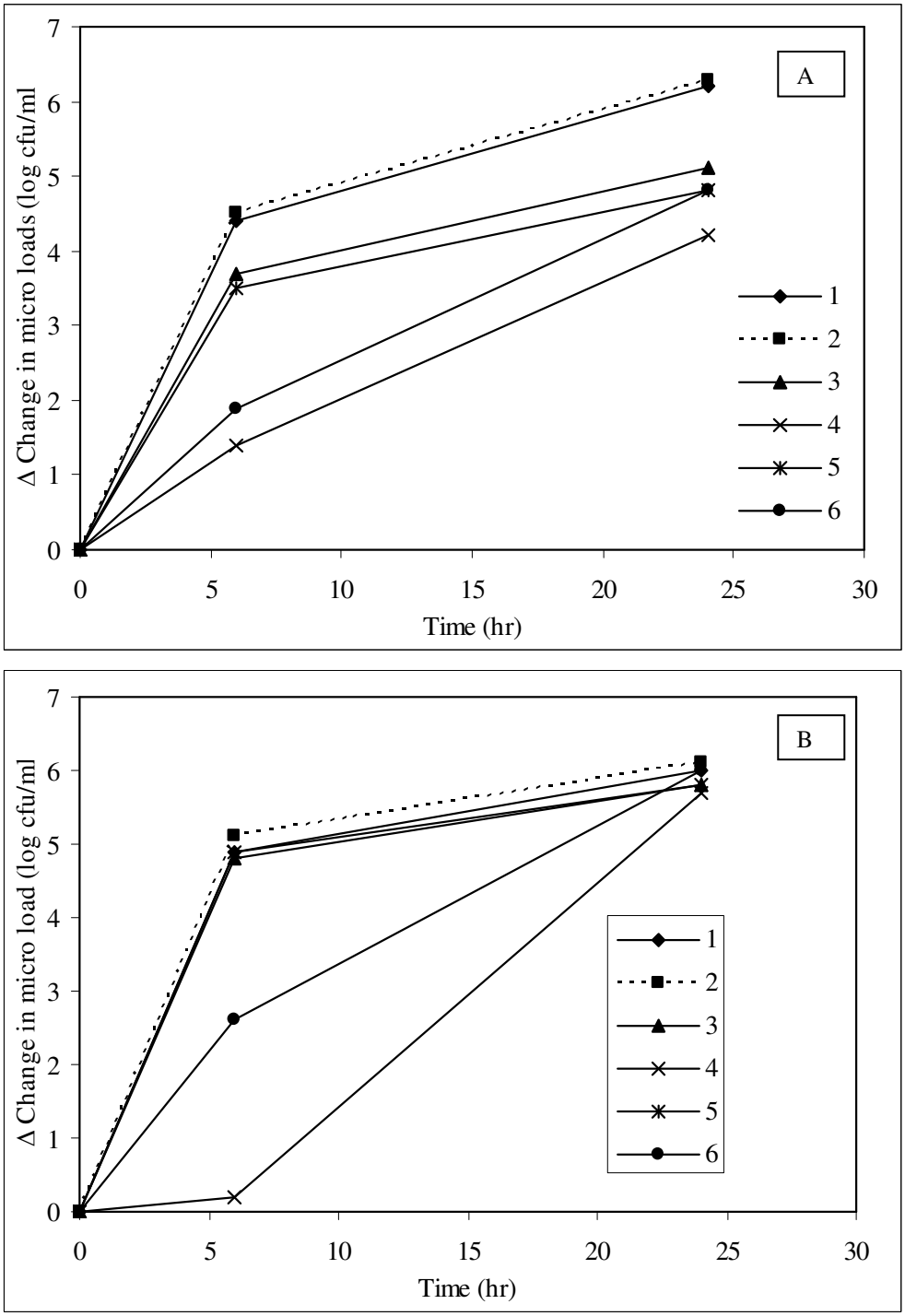


Figure 6.3. Change of microbial counts (log_{10} cfu/mL) of *E. coli* in reaction mixtures containing 200 μM H_2O_2, 1188 U lactoperoxidase/disc (this activity was reached by using 454 μg/disc (A: 1st trial) or 788 μg/disc (B: 2nd trial) enzyme for different LP preparations) and different concentrations of KSCN. Composition of reaction mixtures; 1: Nutrient broth (NB) + *E. Coli* (E) + sterile deionized water (W); 2: NB + E + Alginate disc (A) + W; 3: NB + E + Lactoperoxidase incorporated alginate disc (L) + Potassium thiocyanate (T) + W; 4: NB + E + L + T(1000 μM) + H (200 μM); 5: NB + E + L + T (2000 μM) + H; 6: NB + E + L + T + H.

6.1.3.2. Effects of LP System in Reaction Mixtures Containing Alginate Films Incorporating LP against *L. innocua*

Alginate films incorporating LP were also evaluated for their ability to inhibit *L. innocua.* As seen in Table 6.5, incubation for 6 hr caused an inhibitory effect against *L. innocua* in the presence of lactoperoxidase-H_2O_2-thiocyanate system containing 200 and 400 μM H_2O_2. The inhibitory effect increased further by increasing H_2O_2 to 800 μM (Figure 6.4). In contrast, rapid growth of *L.innocua* was observed in these reaction mixtures between 6 and 24 hr of incubation, with the exception of the 2nd trial reaction mixture containing 800 μM H_2O_2. In the 2nd trial conducted with a different batch of LP, the inhibition in the presence of 800 μM H_2O_2 lasted longer and continued even at the end of 24 hr. Thus, the number of *L. innocua* in this reaction mixture reduced to the lowest level reached in this study. As given in Table 6.6, the H_2O_2 residue was still present in this reaction mixture at the end of 24 hr incubation period. It seems that the remaining H_2O_2 contributed to the long lasting antimicrobial effect of reaction mixture 6 of the 2nd trial.

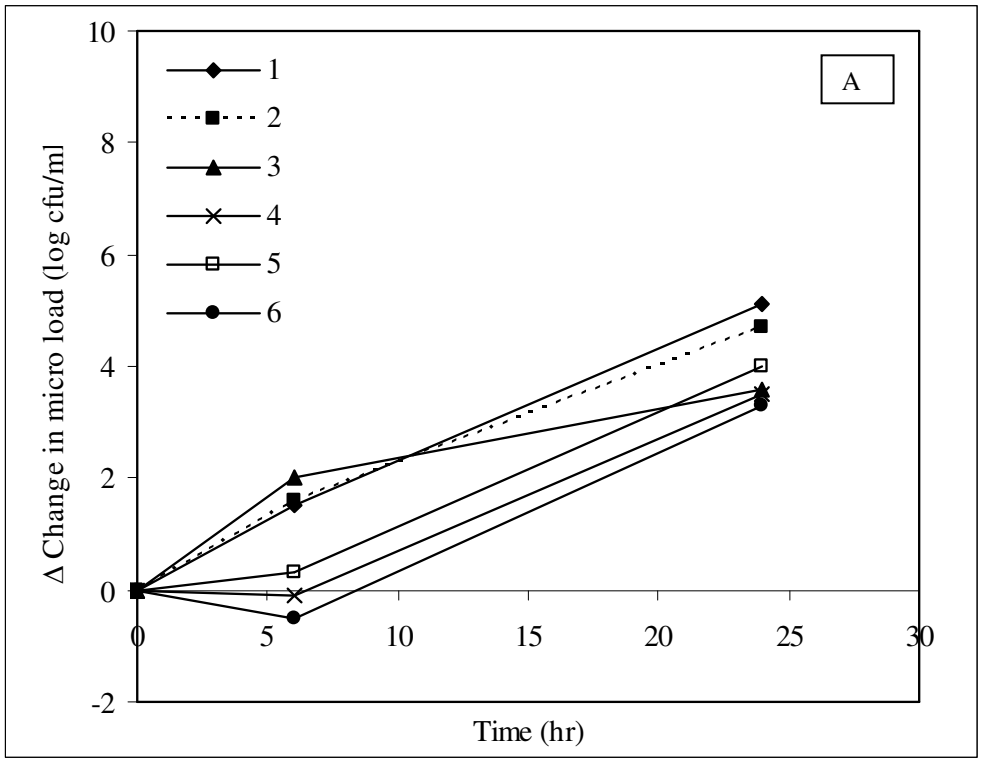
Table 6.5. *L. innocua* counts in reaction mixtures having different concentrations of H_2O_2 during 24 hr incubation at 37 °C.

	No	Total LP activity in discs (U/cm^2)[1]	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	*L. innocua* counts (log_{10} cfu/mL)		
					Incubation time at 37 °C (hr)		
					0	**6**	**24**
1st Trial	1[2]	-	-	-	4.1[c]	5.6[b]	9.2[a]
	2[3]	-	-	-	4.2[c]	5.8[b]	8.9[a]
	3	900(459)[4]	4000	-	5.3[c]	7.3[b]	8.9[a]
	4	900(459)	4000	200	5.3[b]	5.2[b]	8.8[a]
	5	900(459)	4000	400	5.0[c]	5.3[b]	9.0[a]
	6	900(459)	4000	800	5.4[b]	4.9[c]	8.7[a]
2nd Trial	1[2]	-	-	-	4.0[c]	6.5[b]	9.0[a]
	2[3]	-	-	-	4.3[c]	7.1[b]	9.1[a]
	3	900(343)[4]	4000	-	4.1[c]	7.2[b]	9.3[a]
	4	900(343)	4000	200	4.6[c]	5.9[b]	9.2[a]
	5	900(343)	4000	400	4.1[b]	3.9[b]	9.2[a]
	6	900(343)	4000	800	4.1[a]	3.1[c]	3.6[b]

[1] LPS activity of films used for 1st trial: 1188 U/disc (660 µg/disc) and for 2nd trial: 1188 U/disc (453µg/disc)
[2] Solution contains only nutrient broth and *L.innocua*
[3] Solution contains nutrient broth, *L. innocua* and discs without lactoperoxidase enzyme
[4] Lactoperoxidase enzyme amount in µg in each disc
[a-c] Row means having a different letter are significantly different (P<0.05).

Table 6.6. The change of H_2O_2 concentration in reaction mixtures during 24 hr incubation at 37 °C.

	No	H_2O_2 concentration (µM)	H_2O_2 concentration (µM)		
			Incubation time at 37 °C (hr)		
			0	**6**	**24**
1st Trial	1	200	88	-	-
	2	400	294	29-88	-
	3	800	294-800	88-294	-
2nd Trial	1	200	88-200	-	-
	2	400	294	29-88	-
	3	800	294-800	294	29



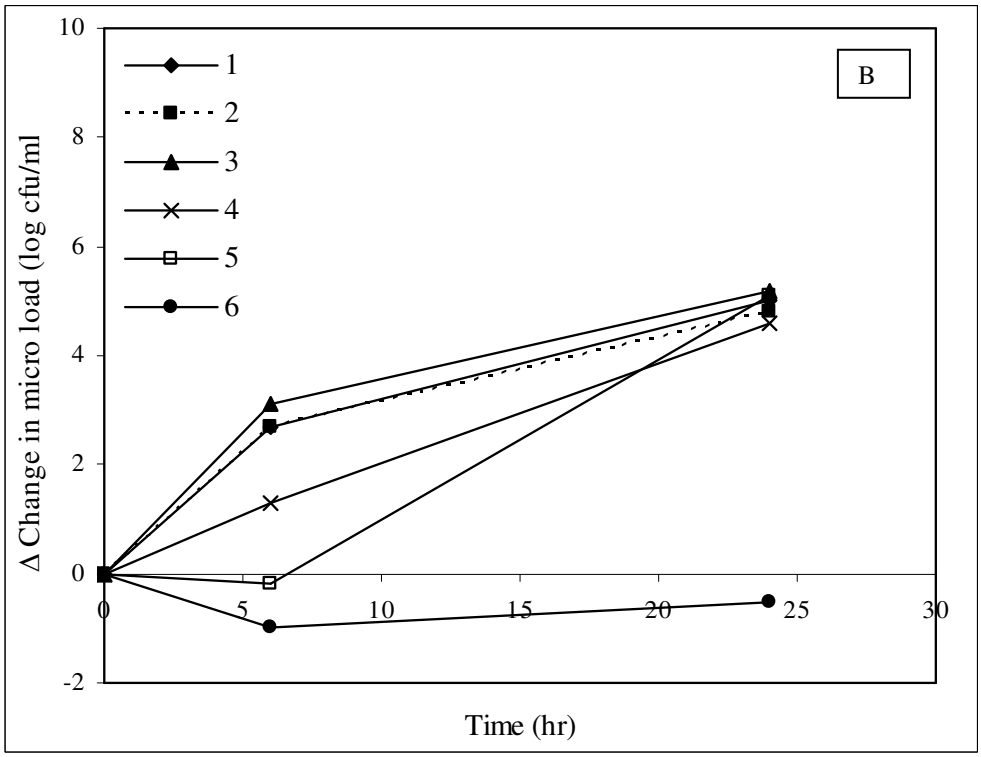


Figure 6.4. Change in the microbial counts (log_{10} cfu/ml) of *L. innocua* in reaction mixtures with different concentrations of H_2O_2 and having 660 µg (A: 1st trial) and 453 µg (B: 2nd trial) of lactoperoxidase in discs in 24 hr (the legends 1, 2, 3, 4, 5, and 6 were the same as those in the Figure 6.2).

In further studies, 200 μM H_2O_2 and 1000 or 2000 μM KSCN concentrations were used for the LP system. As summarized in Table 6.7, the antimicrobial effect lasted up to the 6th hr of incubation. In this time period, the LP system showed a bacteriostatic effect on *L. innocua*. In contrast, between 6th and 24th hrs of incubation there was almost no antimicrobial effect on the bacteria and this caused a rapid microbial growth (Figure 6.5.). The results of this study clearly showed the higher susceptibility of *L. innocua* to LP system than *E. coli*. The LP system is particularly effective on *L. innocua*, in the first 6th hr of incubation. In both cases the antimicrobial effect of the LP system is mostly short lived and rarely exceeded 6 hr. However, it should be noted that the reaction mixtures contain significant number of bacteria which is hard to find in real food applications. In fact, in a food application, the expectation from LP antimicrobial system would be the inhibition of limited number of pathogenic bacteria originated from contamination. The duration of antimicrobial effect of lactoperoxidase system depends on kinetic properties of the enzyme, initial concentrations of hydrogen peroxide and thiocyanate, and catalase activity of the food product. Thus, it is essential to use suitable concentrations of each ingredient during food applications and choose food with low catalase activity at the surface.

Table 6.7. *L. innocua* counts in reaction mixtures having different concentrations of KSCN during 24 hr incubation at 37 °C.

	No	Total LP activity in discs (U/cm^2)[1]	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	*L. innocua* counts (log_{10} cfu/mL)		
					Incubation time at 37 °C (hr)		
					0	6	24
1st Trial	1[2]	-	-	-	3.0[c]	5.4[b]	8.8[a]
	2[3]	-	-	-	3.0[c]	5.5[b]	8.8[a]
	3	900 (363)[4]	1000	-	3.8[c]	6.3[b]	8.9[a]
	4	900 (363)	1000	200	3.5[c]	4.8[b]	8.9[a]
	5	900 (363)	2000	-	3.5[c]	6.2[b]	9.0[a]
	6	900 (363)	2000	200	3.5[c]	4.5[b]	8.9[a]
2nd Trial	1[2]	-	-	-	3.1[c]	5.5[b]	8.8[a]
	2[3]	-	-	-	3.1[c]	5.7[b]	8.6[a]
	3	900 (343)[4]	1000	-	3.2[c]	5.9[b]	9.3[a]
	4	900 (343)	1000	200	3.8[c]	4.7[b]	9.1[a]
	5	900 (343)	2000	-	3.3[c]	6.2[b]	9.1[a]
	6	900 (343)	2000	200	3.4[b]	3.9[b]	9.1[a]

[1] LPS activity of films used for 1st trial:1188 U/disc (479 µg/disc) and for 2nd trial: 900 U/cm^2 (453 µg/disc)
[2] Solution contains only nutrient broth and *L. innocua*
[3] Solution contains nutrient broth, *L. innocua* and discs without lactoperoxidase enzyme
[4] Lactoperoxidase enzyme amount in µg in each disc
[a-c] Row means having a different letter are significantly different (P<0.05).

Table 6.8. The change of H_2O_2 concentration in reaction mixtures during 24 hr incubation at 37 °C.

	No	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	H_2O_2 concentration (µM)		
				Incubation time at 37 °C (hr)		
				0	6	24
1st Trial	1	1000	200	88-200	29	-
	2	2000	200	88-200	0-29	-
2nd Trial	1	1000	200	88-200	29-88	-
	2	2000	200	88-200	29-88	-

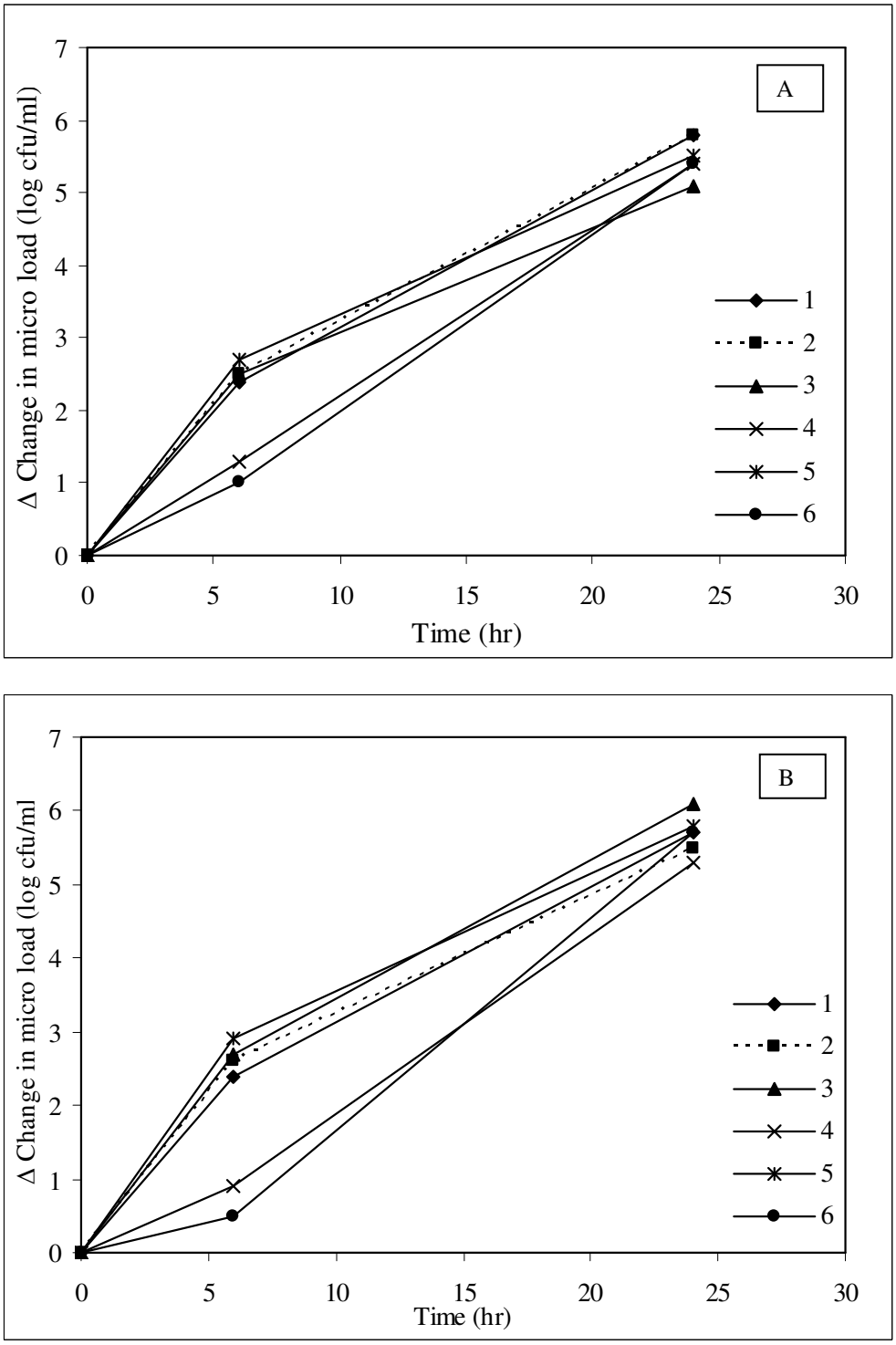


Figure 6.5. Change in the microbial counts (log_{10} cfu/ml) of *L. innocua* in reaction mixtures with different concentrations of KSCN and having 479 µg (A: 1st trial) and 453 µg (B: 2nd trial) of lactoperoxidase in discs in 24 hr (the legends 1, 2, 3, 4, 5, and 6 were the same as those in the Figure 6.3).

6.1.3.3. Effects of LP System in Reaction Mixtures Containing Alginate Films Incorporating LP against *P. fluorescens*

Effect of the LP incorporated alginate films on inhibition of *P. fluorescens* was tested under the same conditions used for the other cultures. The antibacterial effect of the alginate film incorporated with 900 U/cm^2 LP on *P. fluorescens* was given in Table 6.9. In the 1st trial, the results showed that all tubes containing 4000 µM thiocyanate and 200, 400, or 800 µM H_2O_2 were effective against the inhibiton of *P. fluorescens*. Particularly at 800 µM H_2O_2, the LP incorporated alginate films showed a considerable inhibition on *P. fluorescens* up to 24th hr of incubation. In the 2nd trial conducted with a different batch of LP, the desired antibacterial effect was seen in the tubes containing 400 and 800 µM H_2O_2 at 6th hr of incubation, but at 24th hr the antibacterial effect was diminished. The H_2O_2 consumption in reaction mixtures of 1st and 2nd trials were different (Table 6.10). Thus, it seems that there are differences between the kinetic properties of different batches of LPs. The differences (almost 1 log) in the initial microbial numbers of reaction mixtures may also contribute to variations in antimicrobial effect of LPs since the amount of organic matter may affect the half-life of reactive antimicrobial products formed by the LP system.

Table 6.9. *P. fluorescens* counts in reaction mixtures having different concentrations of H_2O_2 during 24 hr incubation at 26 °C.

	No	Total LP activity in discs (U/cm^2)[1]	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	*P. fluorescens* counts log_{10} (cfu/mL)		
					Incubation time at 26 °C (hr)		
					0	6	24
1st Trial	1[2]	-	-	-	3.1[c]	4.0[b]	8.4[a]
	2[3]	-	-	-	2.7[b]	4.3[b]	8.2[a]
	3	900 (301)[4]	4000	-	3.6[c]	4.8[b]	9.1[a]
	4	900 (301)	4000	200	2.8[b]	2.5[c]	8.7[a]
	5	900 (301)	4000	400	2.5[b]	2.0[c]	4.0[a]
	6	900 (301)	4000	800	3.4[a]	0.7[b]	0.7[b]
2nd Trial	1[2]	-	-	-	4.0[c]	5.1[b]	8.7[a]
	2[3]	-	-	-	4.1[c]	5.5[b]	8.2[a]
	3	900 (260)[4]	4000	-	4.9[c]	6.5[b]	9.0[a]
	4	900 (260)	4000	200	5.3[c]	6.4[b]	9.0[a]
	5	900 (260)	4000	400	4.8[b]	4.3[c]	9.1[a]
	6	900 (260)	4000	800	4.1[b]	2.4[c]	8.8[a]

[1] LPS activity of films used for 1st trial: 1188 U/disc (398µg/disc) and for 2nd trial: 1188 U/disc (344 µg/disc)
[2] Solution contains only nutrient broth and *P.fluorescens*
[3] Solution contains nutrient broth, *P.fluorescens* and discs without lactoperoxidase enzyme
[4] Lactoperoxidase enzyme amount in µg in each disc
[a-c] Row means having a different letter are significantly different (P<0.05).

Table 6.10. The change of H_2O_2 concentration in reaction mixtures during 24 hr incubation at 26 °C.

	No	H_2O_2 concentration (µM)	H_2O_2 concentration (µM)		
			Incubation time at 26 °C (hr)		
			0	6	24
1. Trial	1	200	88-200	29-88	-
	2	400	294-400	88-294	29-88
	3	800	294-800	294-800	88-294
2. Trial	1	200	88-200	-	-
	2	400	294-400	88	-
	3	800	294-800	88-294	-

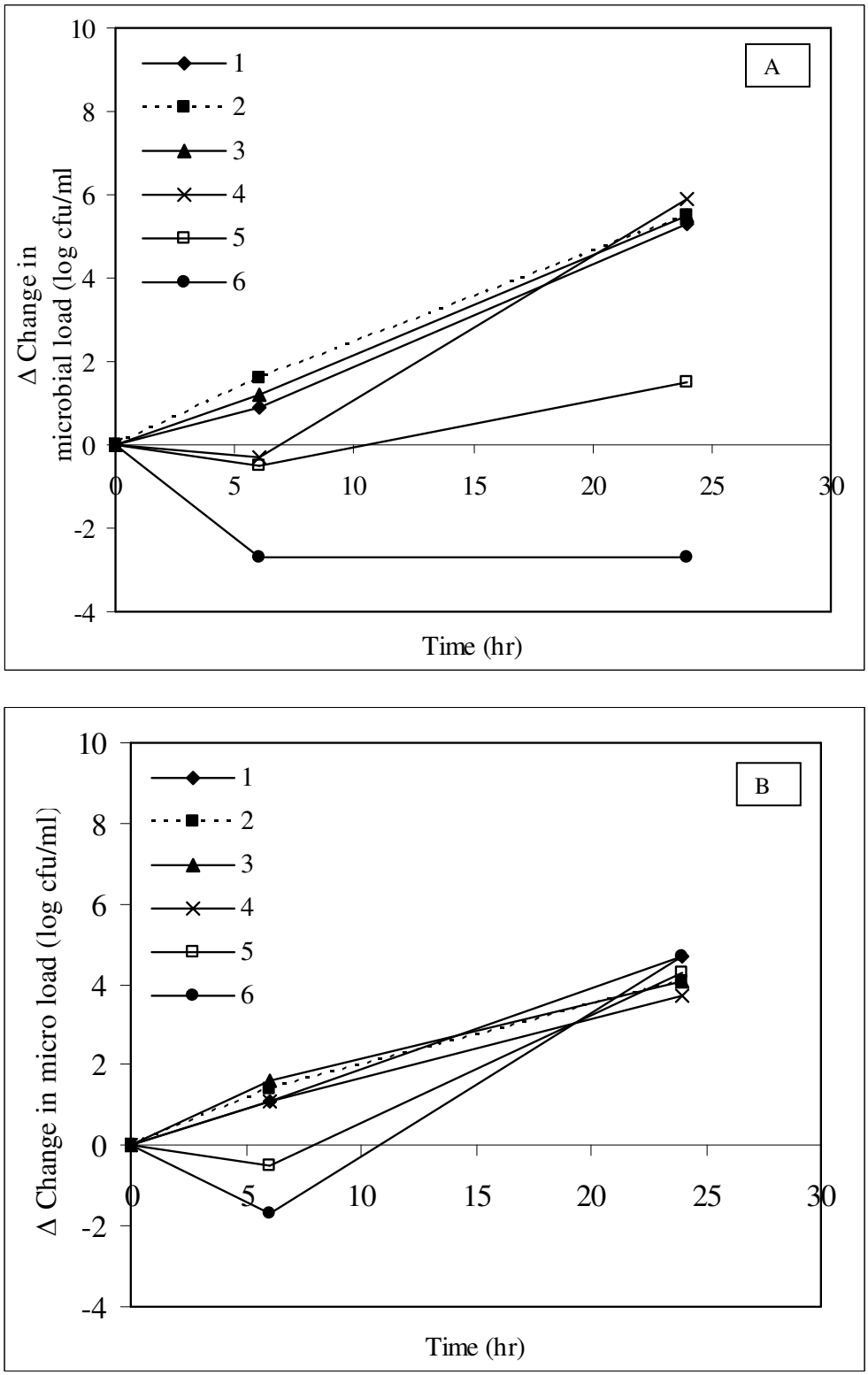


Figure 6.6. Change in the microbial counts (log_{10} cfu/ml) of *P. fluorescens* in reaction mixtures with different concentrations of H_2O_2 and having 398 µg (A: 1st trial) and 344 µg (B: 2nd trial) of lactoperoxidase in discs in 24 hr (the legends 1, 2, 3, 4, 5, and 6 were the same as those in Figure 6.2).

The antimicrobial effect of LP system on *P. fluorescens* was also tested at 1000 and 2000 µM thiocyanate and 200 µM H_2O_2 (Table 6.11). As seen in Figure 6.7 the reaction mixture containing LP incorporated alginate films and KSCN and H_2O_2 showed considerable antimicrobial effect on *P. fluorescens* in the first 6^{th} hr of incubations. Then the antimicrobial effect was diminished and rapid growth occurred between 6^{th} and 24^{th} hrs of incubation. In reaction mixtures containing KSCN but lacking H_2O_2, the microbial growth occurred more rapidly than the other reaction mixtures, including the controls. This result suggested the activatory activity of KSCN, but it occurred only with *P. fluorescens*.

Table 6.11. *P. fluorescens* counts in reaction mixtures having different concentrations of thiocyanate and H_2O_2 during 24 hr incubation at 26 °C.

	No	Total LP activity in discs (U/cm^2)[1]	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	*P. fluorescens* counts (log_{10} cfu/mL)		
					Incubation time at 26 °C (hr)		
					0	6	24
1st Trial	1[2]	-	-	-	2.5[c]	3.2[b]	8.4[a]
	2[3]	-	-	-	2.6[b]	2.9[b]	8.2[a]
	3	900 (413)[4]	1000	-	3.3[c]	5.3[b]	8.3[a]
	4	900 (413)	1000	200	2.7[b]	1.5[b]	5.6[a]
	5	900 (413)	2000	-	3.0[c]	5.5[b]	8.9[a]
	6	900 (413)	2000	200	2.7[c]	1.4[b]	7.1[a]
2nd Trial	1[2]	-	-	-	3.6[b]	3.6[b]	7.5[a]
	2[3]	-	-	-	3.6[c]	4.7[b]	7.8[a]
	3	900 (260)[4]	1000	-	3.8[c]	4.8[b]	8.9[a]
	4	900 (260)	1000	200	3.7[b]	3.4[b]	8.6[a]
	5	900 (260)	2000	-	3.7[c]	4.7[b]	8.9[a]
	6	900 (260)	2000	200	3.7[b]	3.1[c]	8.5[a]

[1] LPS activity of films used for 1st trial: 1188 U/disc (545 µg/disc) and for 2nd trial: 1188 U/disc (398 µg/disc)
[2] Solution contains only nutrient broth and *P. fluorescens*
[3] Solution contains nutrient broth, *P. fluorescens* and discs without lactoperoxidase enzyme
[4] Lactoperoxidase enzyme amount in µg in each disc
[a-c] Row means having a different letter are significantly different (P<0.05).

Table 6.12. The change of H_2O_2 concentration in reaction mixtures during 24 hr incubation at 26 °C.

	No	Thiocyanate conc. (µM)	H_2O_2 conc. (µM)	H_2O_2 concentration (µM)		
				Incubation time at 26 °C (hr)		
				0	6	24
1st Trial	1	1000	200	88-200	-	-
	2	2000	200	88-200	0-29	-
2nd Trial	1	1000	200	88-200	88	-
	2	2000	200	88-200	88	-

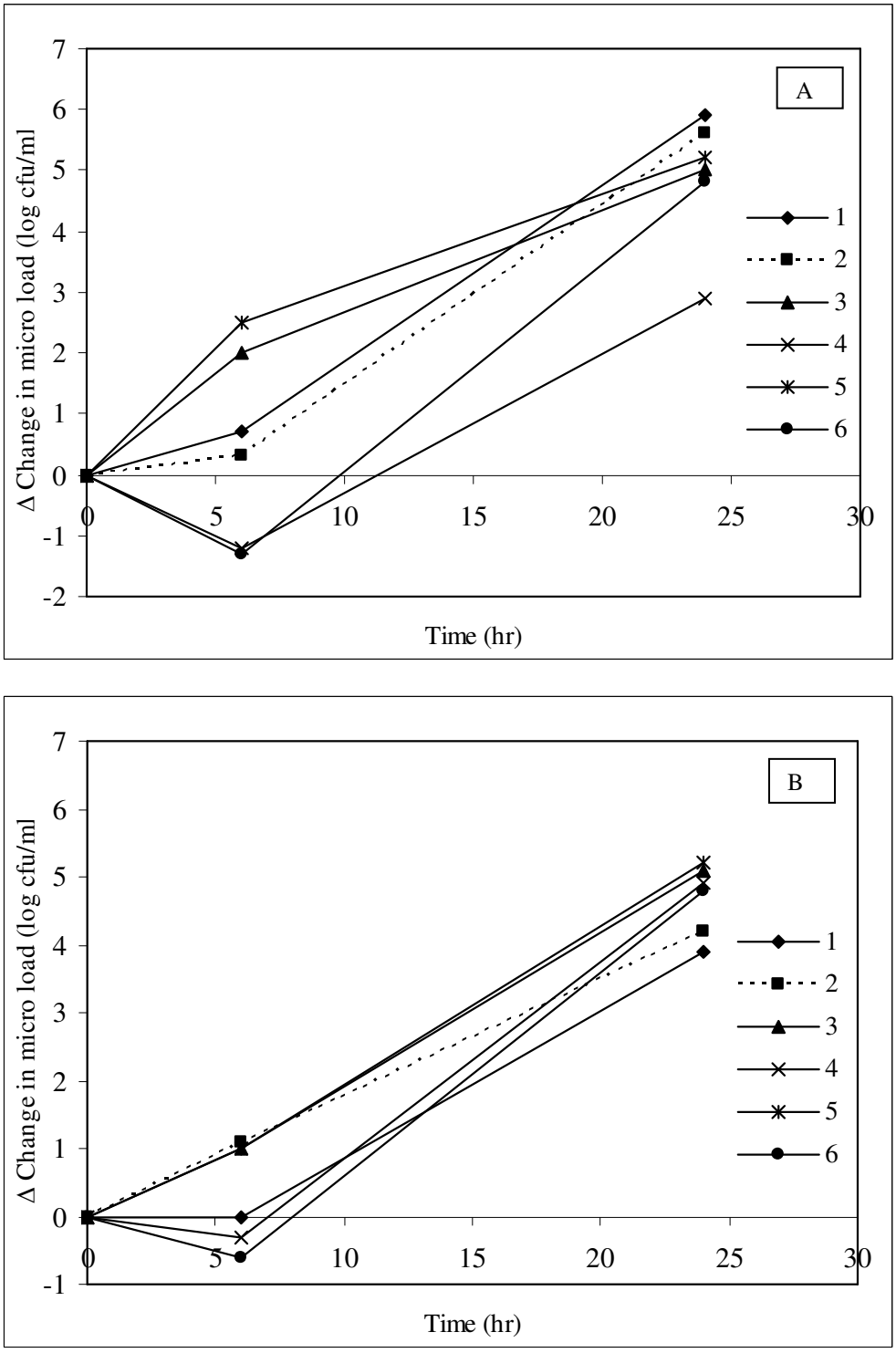


Figure 6.7. Change in the microbial counts (log_{10} cfu/ml) of *P. fluorescens* in reaction mixtures with different concentrations of KSCN and having 545 µg (A: 1st trial) and 398 µg (B: 2nd trial) of lactoperoxidase in discs in 24 hr (the legends 1, 2, 3, 4, 5, and 6 were the same as those in Figure 6.3).

6.1.4. Test of Developed LP Incorporated Alginate Films on Refrigerated Fresh Calamari

In this study, the developed antimicrobial system based on LP system was tested in a real food application by using on refrigerated raw calamari. The antimicrobial film system was formed by dipping rings of calamari into alginate film forming solutions containing 2.2 mg LP per g solution (The activity of LP was 2921 U/mg) and 4000 µM KSCN. The calamari rings were then dipped into 0.3 M $CaCl_2$ to cross-link the film at their surface and placed into a 45 mL solution containing 4000 or 8000 µM H_2O_2. The uncoated controls and alginate coated controls were placed into 45 mL of sterile distilled water instead of H_2O_2 solution. As seen in Table 6.13, the application of LP system at different H_2O_2 concentrations did not change the initial microbial load of calamari samples considerably. However, the beneficial effects of LP system on microbial load were observed clearly during cold storage of samples. In calamari coated with LP and KSCN incorporated alginate films and cold stored in the presence of 4000 and 8000 µM H_2O_2, the microbial load did not change for almost 2 and 3 days, respectively. In contrast, in uncoated and coated control calamari the microbial load of samples were 1 decimal over 6 $\log_{10}$ cfu/g, considered as a limit in the shelf-life determination studies.

Table 6.13. Effect of LP incorporated alginate films on total viable counts of coated calamari rings during refrigerated storage.

Treatments	Total viable count (log cfu/g)					
	Storage period (days)					
	0	1	2	3	5	7
Uncoated calamari (stored in water)						
	$5.12\pm0.04^{e,x}$	$7.16\pm0.13^{d,x}$	$7.57\pm0.07^{c,x}$	$8.09\pm0.27^{b,x}$	$8.90\pm0.11^{a,x}$	$8.81\pm0.19^{a,x}$
Control calamari coated with alginate film (stored in water)						
	$5.08\pm0.15^{d,x}$	$7.30\pm0.08^{c,x}$	$7.49\pm0.23^{c,x}$	$8.32\pm0.16^{b,x}$	$8.90\pm0.14^{a,x}$	$9.10\pm0.04^{a,x}$
Calamari coated with alginate film incorporated with LP and KSCN (stored in 4000 µM H_2O_2 solution)						
	$4.91\pm0.14^{c,xy}$	$5.04\pm0.23^{c,y}$	$5.00\pm0.65^{c,y}$	$6.55\pm0.34^{b,y}$	$7.81\pm1.05^{ab,xy}$	$8.57\pm0.34^{a,x}$
Calamari coated with alginate film incorporated with LP and KSCN (stored in 8000 µM H_2O_2 solution)						
	$4.68\pm0.15^{b,y}$	$4.57\pm0.22^{b,z}$	$4.30\pm0.40^{b,y}$	$4.58\pm1.36^{b,z}$	$6.39\pm1.04^{ab,y}$	$6.75\pm1.12^{a,y}$

[a-e] Row means having a different letter are significantly different (P<0.05).
[x-z] Column means having a different letter are significantly different (P<0.05).

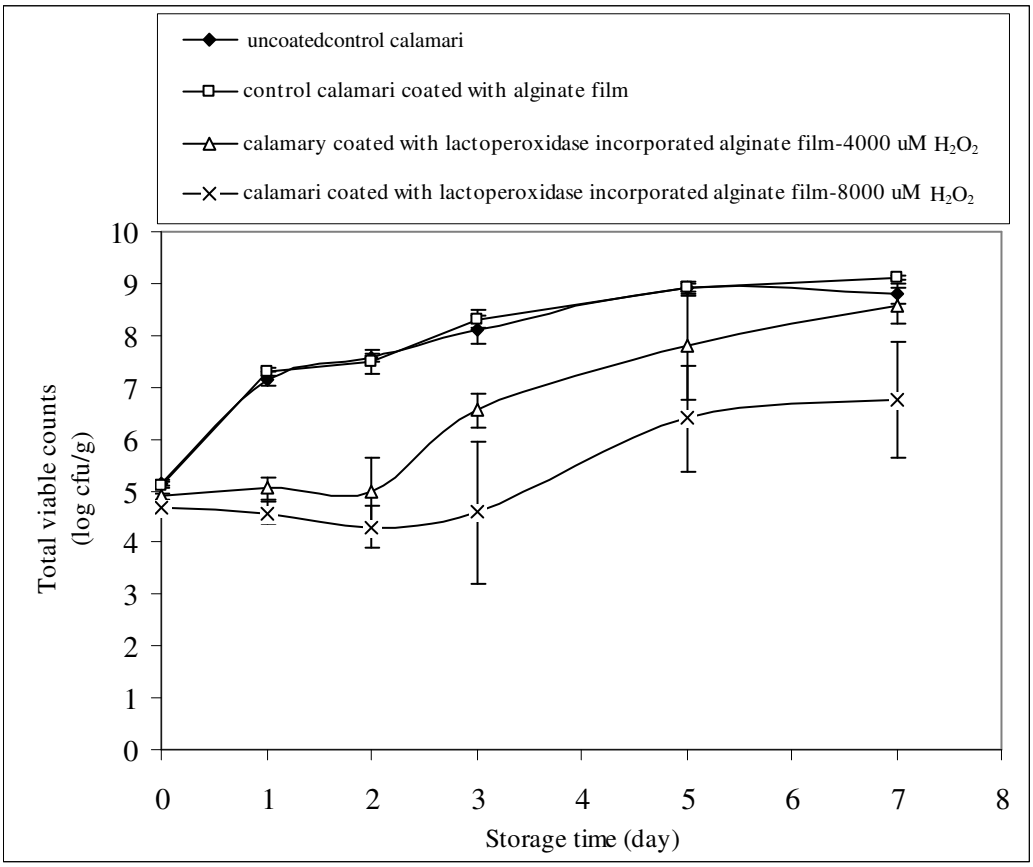


Figure 6.8. The change in the total viable counts of the calamari rings coated with alginate films incorporating LP during storage.

6.2. Alginate Films Incorporated with Lactic Acid Bacteria

6.2.1. Comparison of *L. casei* and *L. delbrueckii* subsp. *lactis* for Hydrogen Peroxide Production at 37 °C

To determine the ability of H_2O_2 production of *L.casei* and *L.delbrueckii* subsp. *lactis*, two cultures were incubated using MRS agar at 37 °C for 24 hr. During incubation, the H_2O_2 production and the turbidity of the growth media were monitored. The results were given in Table 6.14. The levels of H_2O_2 produced by *L. delbrueckii* subsp. *lactis* ranged from 1 to 10 mg/L between 6 and 24 hrs. Increase in turbidity by *L.casei* took place rapidly in contrast to *L. delbrueckii* subsp. *lactis*. The microbial loads of *L. casei* and *L. delbrueckii* subsp. *lactis* at 37 °C for 24 hr incubation were shown in Figure 6.9. According to these results, *L. delbrueckii* subsp. *lactis* was selected for the

next studies due to its ability of H_2O_2 production. The maximum amount of H_2O_2 was produced at the end of the exponential growth.

Table 6.14. The amount of hydrogen peroxide and change in the optical density by *L.casei* and *L.delbrueckii* subsp. *lactis* at 37 °C during 24 hr incubation.

Time (hour)	***L. casei***		***L. delbrueckii* subsp. *lactis***	
	H_2O_2 (mg/L)	**OD $_{650\ nm}$**	**H_2O_2 (mg/L)**	**OD $_{650\ nm}$**
0	0	0.0426	0	-0.0012
3	0	0.1647	0	0.0093
4	0	0.3339	0	-
5	0	0.5056	0	0.0157
6	0	0.7957	1	0.0365
7	0	1.1127	3	0.0608
8	0	1.4875	3	0.0981
9	0	1.9528	10	0.1546
24	0	2.5127	10	1.491

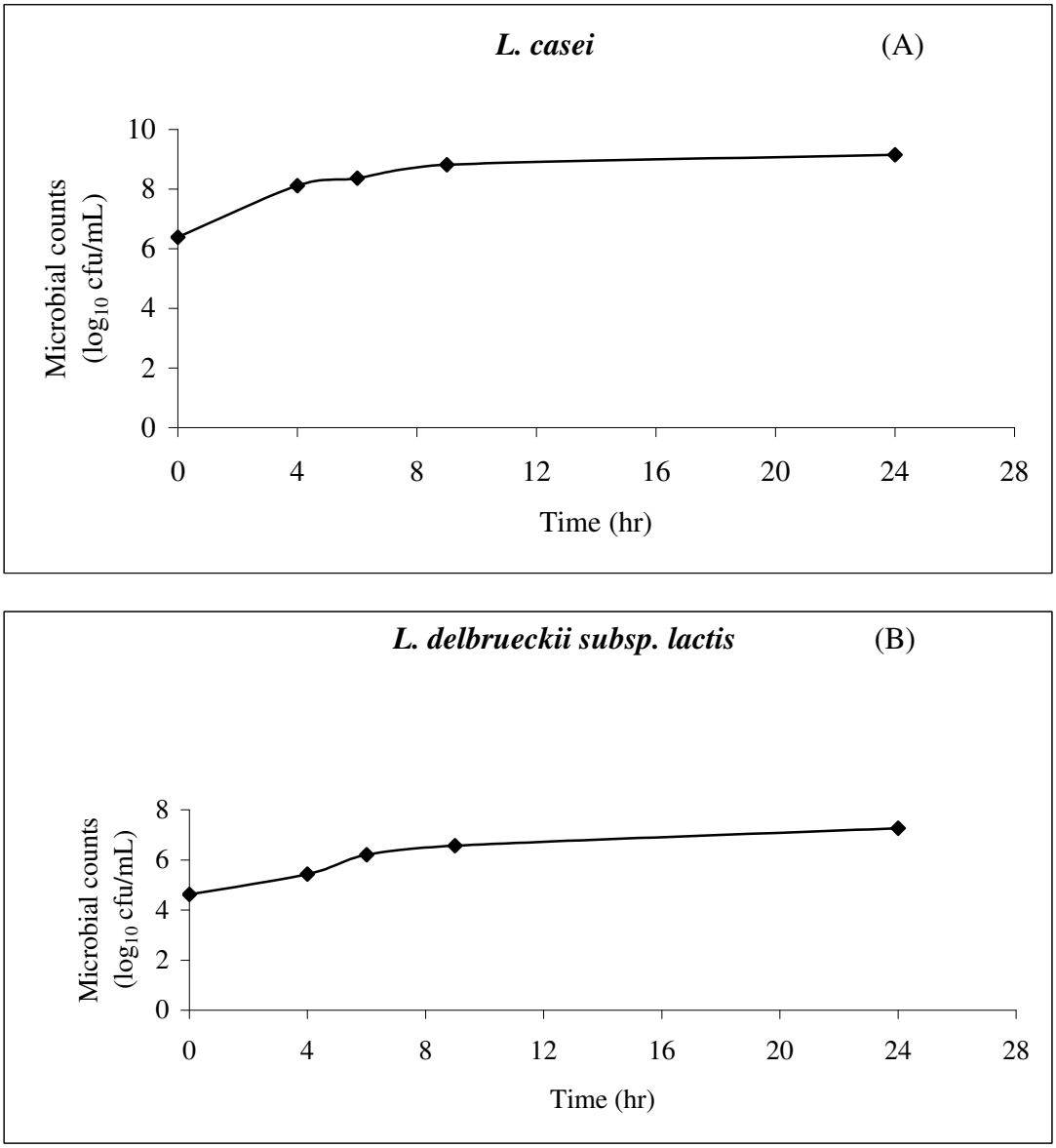


Figure 6.9. The microbial growth of *L. casei* (A) and *L. delbrueckii* subsp. *lactis* (B) at 37 °C.

6.2.2. H_2O_2 and Lactic Acid Production of *L. delbrueckii* subsp. *lactis* and *L. plantarum* at Different Storage Temperatures

Lactic acid bacteria could be used as protective cultures because they become dominant on microflora during storage and also they produce antimicrobial products such as lactic acid, H_2O_2, bacteriocin, etc. to inhibit the growth of other microorganisms. One of the most effective mechanisms that the bacteria utilize to form the inhibitory action is lactic acid production. *L.delbrueckii* subsp.*lactis* bacterium can produce lactic acid as well as H_2O_2 at storage temperatures (Zalan et al. 2005). Thus, it

may have a potential for incorporation into edible films as protective bacteria. Another advantage of *L. delbrueckii* subsp. *lactis* is the production of lactic acid in lower amounts and especially at the stationary phase. This is of great significance in terms of not altering the products' sensorial quality at the early stages of storage.

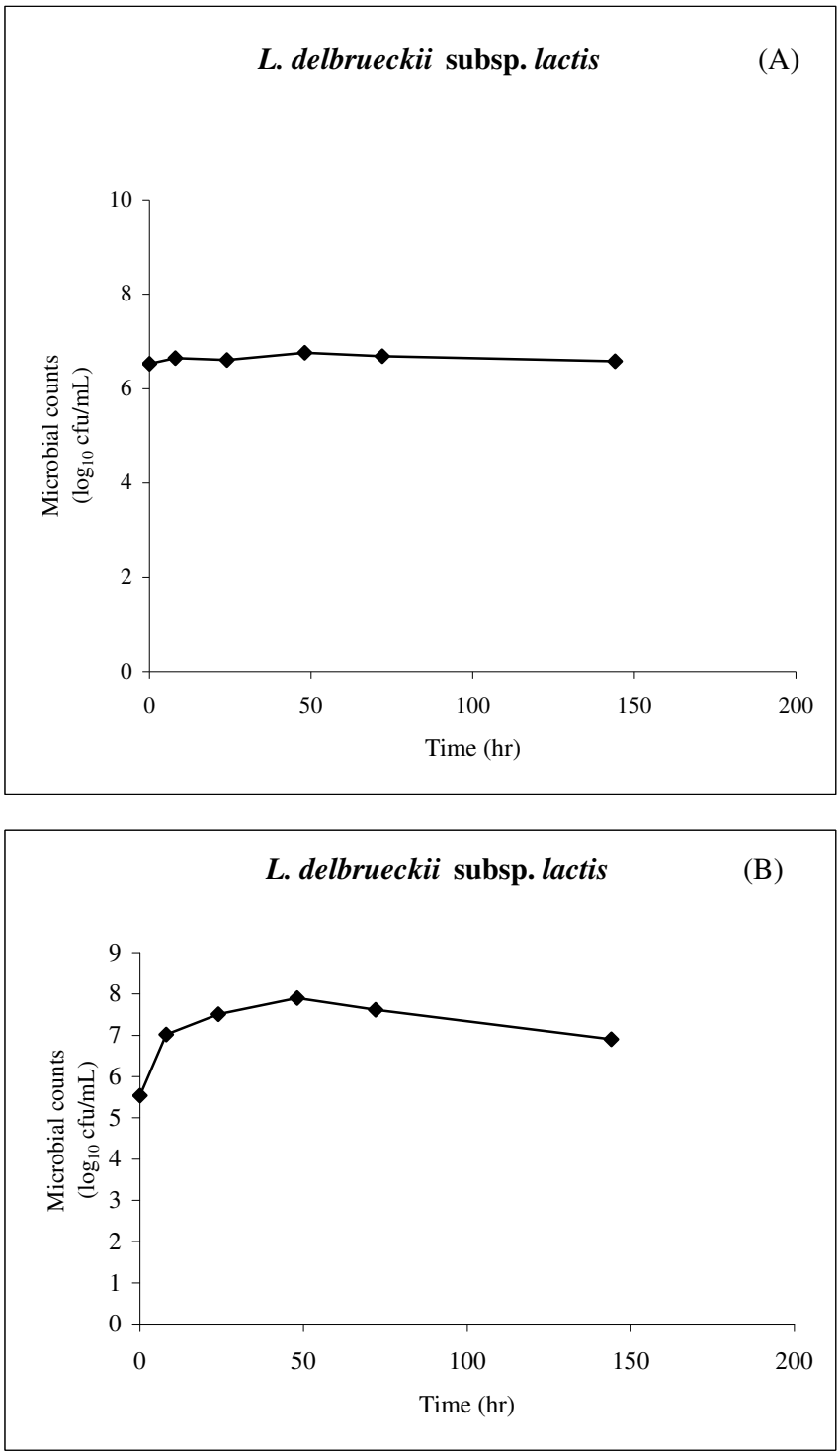


Figure 6.10. The growth curve of *L.delbrueckii subsp. lactis* at 4℃ (A) and 23℃ (B).

The growth curves at 4 °C and 23 °C as well as lactic acid and H_2O_2 concentrations and pH values of growth medium during incubation were given in Figure 6.10 and Table 6.15, respectively. According to the obtained results, it was clear that at 4°C *L. delbrueckii* subsp. *lactis* cannot multiply and produce lactic acid and H_2O_2. On the other hand, at 23 °C, there was an increase in the microbial load, and bacteria began producing H_2O_2 and lactic acid after 24 h and 144 h, respectively. The bacteria were unable to produce L-lactic acid and it formed only D-lactic acid under the studied conditions. On the other hand, the required time at 23 °C to reach 10 mg H_2O_2/L was almost 5-fold longer than that necessary to obtain the same concentration of H_2O_2 at 37 °C (see Table 6.14). Thus, it is clear that the increase in temperature reduced the time period to reach the stationary phase at which most of the antimicrobial products are produced by the bacteria. The results of this study showed that the *L.delbrueckii subsp. lactis* is not an extensive producer of H_2O_2 and lactic acid at the studied conditions. However, the production of both of these antimicrobial products may be effective on growth of pathogenic bacteria when *L. delbrueckii subsp. lactis* was incorporated into alginate films for antimicrobial packaging. It is also possible that this bacteria show protective effect by dominating over other bacteria by occupying of food surface and minimizing nutritive factors.

Table 6.15. The H_2O_2 and lactic acid production ability of *L. delbrueckii* subsp. *lactis* and pH change at different storage temperatures.

Time (hr)	**Incubation Temperature**							
	4 °C				**23 °C**			
	pH	**H_2O_2 (mg/L)**	**D-Lactic acid (mg/L)**	**L-Lactic acid (mg/L)**	**pH**	**H_2O_2 (mg/L)**	**D-Lactic acid (mg/L)**	**L-Lactic acid (mg/L)**
0	6.59	0	0	0	6.57	0	0	0
8	6.60	0	0	0	6.58	0	0	0
24	6.61	0	0	0	6.45	3	0	0
48	6.60	0	0	0	6.14	10	0	0
72	6.60	0	0	0	5.93	10	0	0
144	6.64	0	0	0	5.72	3-10	50	0

The growth curves of *L. plantarum* at 4° and 23 °C were given in Figure 6.11. The bacteria cannot grow at 4 °C, but it grows at 23 °C and produces both D- and L-forms of lactic acid (Table 6.16.). At both 4° and 23 °C, the bacteria could not produce

any H_2O_2. However, the significant amount of lactic acid production by the bacteria showed that it could be used as a protective bacterium particularly at temperature abuse conditions.

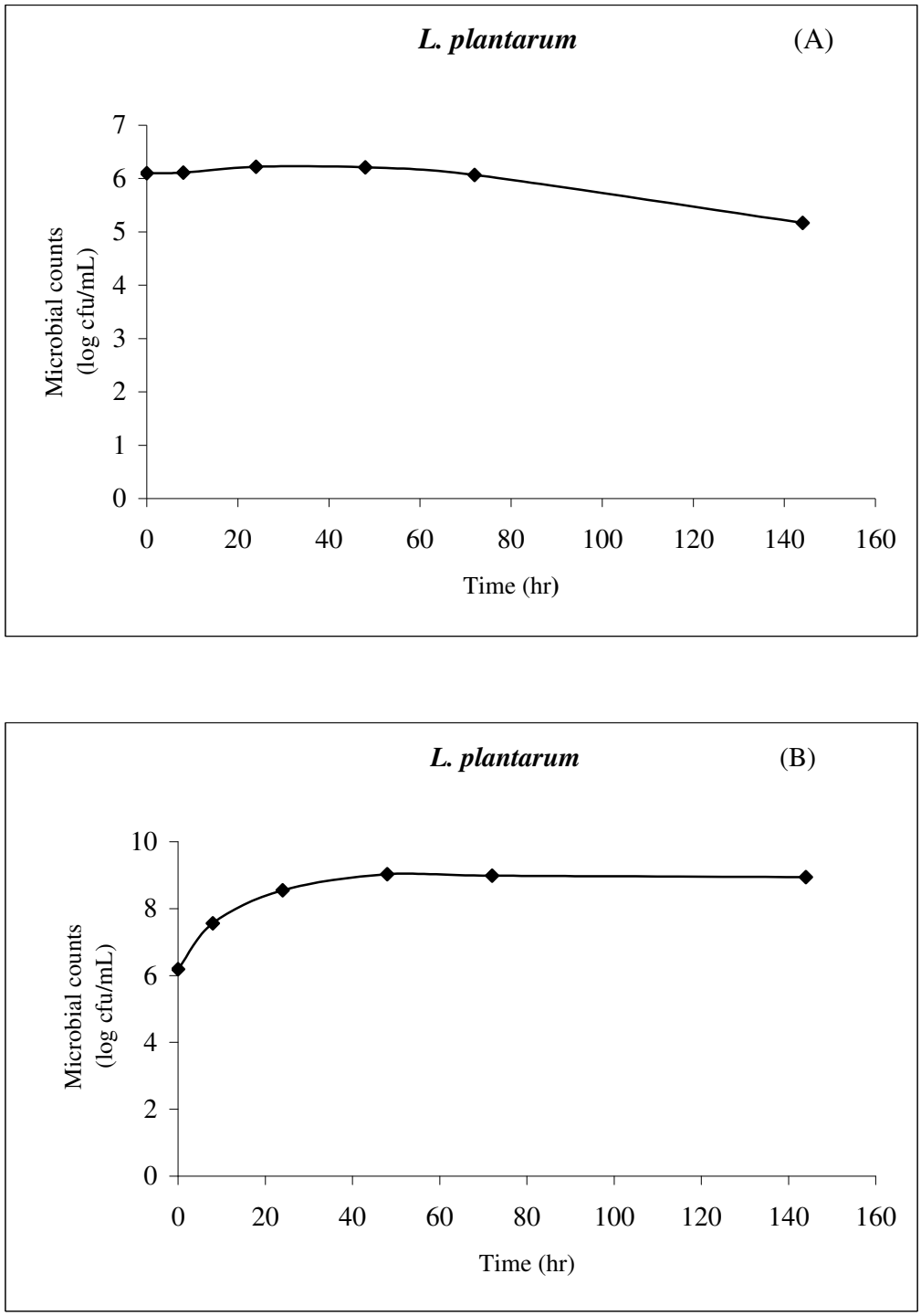


Figure 6.11. The growth curve of *L. plantarum* at 4 °C (A) and 23 °C (B).

Table 6.16. The H_2O_2 and lactic acid production ability of *L. plantarum* and pH change at different storage temperatures.

Time (hour)	Incubation Temperature							
	4ºC				23ºC			
	pH	H_2O_2 (mg/L)	D-Lactic acid (mg/L)	L-Lactic acid (mg/L)	pH	H_2O_2 (mg/L)	D-Lactic acid (g/L)	L-Lactic acid (g/L)
0	6.78	0	0	0	6.78	0	0	0
8	6.80	0	0	0	6.71	0	0	0
24	6.82	0	0	0	6.33	0	0.048	0.307
48	6.83	0	0	0	5.80	0	0.495	0.663
72	6.86	0	0	0	5.46	0	0.735	0.953
144	6.86	0	0	0	4.87	0	1.695	2.569

6.2.3. Incorporation of Lactic Acid Bacteria into Alginate Films

The lactic acid bacteria in the films may dominate the potential pathogenic contaminants at the food surface and act as a protective mechanism in case of temperature abuse conditions. Therefore, *L. plantarum* and *L. delbrueckii* subsp. *lactis* tested for some of their properties which were stated above were incorporated into alginate films following preparation and lyophilization by the methods described in the materials and methods section (Chapter 5, section 5.2.2.4).

6.2.4. Determination of the Number of Free and Immobilized Lactic Acid Bacteria in Alginate Films

This work was conducted to determine both the number of free and entrapped immobilized lactic acid bacteria in the films. For this purpose, the films were kept in peptone water for some time to release free bacteria. The number of immobilized bacteria, on the other hand, was determined by homogenization of the films kept in peptone water. As shown in Table 6.17 and 6.18, the number of lactic acid bacteria immobilized in the film was at least 1000-fold higher than that of the free bacteria released from the film. However, there were also considerable numbers of free lactic acid bacteria in the films and these bacteria can diffuse into food surface. The increase of incorporated lactic acid bacteria increased the number of free and immobilized lactic acid bacteria in the films. However, to obtain 1 decimal increase in the number of free

and immobilized bacteria, the incorporated lactic acid bacteria content should be increased 6 fold (from 2 to 12 mg/g film forming solution).

Table 6.17. The free and immobilized lactic acid bacteria counts in alginate films incorporating different amounts of *L. delbrueckii* subsp. *Lactis.*

Amount of lactic acid bacteria incorporated into the film (mg/g film forming solution)[a]	**Lactic acid bacteria counts (free in the film) (log_{10} cfu/g film)**	**Lactic acid bacteria counts (immobilized in the film) (log_{10} cfu/g film)**
2	2.98	6.16
4	3.61	6.84
6	3.74	6.76
8	3.18	7.10
10	3.76	7.04
12	3.98	7.25

[a]the count of *L.debruekii* subsp. *lactis* in the preparate was 3.0×10^9 cfu/g.

Table 6.18. The free and immobilized lactic acid bacteria counts in the alginate films incorporating different amounts of *L. plantarum.*

Amount of lactic acid bacteria incorporated into the film (mg/g film forming solution)[a]	**Lactic acid bacteria counts (free in the film) (log_{10} cfu/g film)**	**Lactic acid bacteria counts (immobilized in the film) (log_{10} cfu/g film)**
2	3.61	7.77
4	4.78	7.62
6	4.77	7.78
8	4.60	8.11
10	4.98	8.29
12	4.85	8.91

*the count of *L.plantarum* in the preparate was 3.27×10^{10} cfu/g.

6.2.5. Determination of the Stability of the Lactic Acid Bacteria Incorporated into the Alginate Film Forming Solution

In this part of the study, the lyophilized lactic acid bacteria were suspended in the alginate film forming solutions, and then the solutions were cast into Petri dishes and stored at 4 °C for 7 days. At different time periods, the film forming solutions were taken, cross-linked with $CaCl_2$ and enumerated for their lactic acid bacteria counts.

Similar to our previous results, the initial number of free lactic acid bacteria in the films was almost 1000-fold less than the number of immobilized bacteria in the films. On the other hand, lactic acid bacteria, either free or immobilized in the films, showed considerable stability in alginate film forming solutions during cold storage. This result has a critical importance for commercial use of alginate films for incorporation of lactic acid bacteria. Based on these results, it could be concluded that the film solution incorporating the lactic acid bacteria for coating food products could be a commercial preparation having minimum 1 week shelf-life.

Table 6.19. The free and immobilized lactic acid bacteria counts (*L. delbrueckii* subsp. *lactis*) in the stored alginate film solution incorporating lactic acid bacteria.

Storage period (day)	**Lactic acid bacteria counts (free in the film) (log_{10} cfu/g film)**	**Lactic acid bacteria counts (immobilized in the film) (log_{10} cfu/g film)**
0	3.13	6.61
1	3.05	6.64
3	3.05	6.73
7	3.02	6.62

Table 6.20. The free and immobilized lactic acid bacteria counts (*L. plantarum*) in the stored alginate film solution incorporating lactic acid bacteria.

Storage period (day)	**Lactic acid bacteria counts (free in the film) (log_{10} cfu/g film)**	**Lactic acid bacteria counts (immobilized in the film) (log_{10} cfu/g film)**
0	4.81	8.72
1	5.01	8.30
3	4.77	8.39
7	4.79	7.75

6.2.6. Determination of the Stability of the Lactic Acid Bacteria in Alginate Powder

The lyophilized lactic acid bacteria could be mixed with appropriate amount of alginic acid in order to obtain a preparate in powder form. The use of this preparate could provide an ease for applications in food industry as well as minimizing the contamination risk which could occur during handling. This experiment was performed

to determine the stability of the lactic acid bacteria in the preparate during storage. The addition of 60 and 120 mg of lactic acid bacteria either *L. delbrueckii* subsp. *lactis* or *L. plantarum* into the alginic acid preparate did not increase the bacteria counts (Table 6.21). During storage, no significant change occurred in the bacteria counts of the both preparates. This result clearly shows the high applicability of alginate films for incorporation of lactic acid bacteria.

Table 6.21. Lactic acid bacteria counts of cold stored alginate powders containing different amounts of *L. delbrueckii* subsp. *lactis.*

Storage period (day)	**Lactic acid bacteria counts in the preparate incorporating 60 mg *L. del*. subsp. *lactis* (log_{10} cfu/g)**	**Lactic acid bacteria couts in the preparate incorporating 120 mg *L. del*. subsp. *lactis* (log_{10} cfu/g)**
0	8.82	9.07
7	9.04	9.04
14	8.36	8.72
28	8.44	8.80
63	8.52	9.12

Table 6.22. Lactic acid bacteria counts of cold stored alginate powders containing different amounts of *L. plantarum.*

Storage period (day)	**Lactic acid bacteria counts in the preparate incorporating 60 mg *L. plantarum* (log_{10} cfu/g)**	**Lactic acid bacteria counts in the preparate incorporating 120 mg *L. plantarum* (log_{10} cfu/g)**
0	9.81	10.08
7	9.79	10.00
14	9.81	9.94
28	9.82	9.85
63	9.81	9.74

6.2.7. Test of Developed Lactic Acid Bacteria Incorporated Alginate Films on Refrigerated Fresh Beef Cubes

In this study, lyophilized preparations of *L. delbrueckii* subsp. *lactis* ($1.0x10^9$ cfu/g preparation) and *L. plantarum* ($3.3x10^{10}$ cfu/g preparation) were incorporated into the alginate film forming solutions. These solutions were then used to coat fresh beef cubes. The main objective of this study was to test whether the selected cultures would

increase lactic acid bacteria load of raw beef. It is also intended to test the viability of incorporated bacteria in the films on beef surface during refrigerated storage. As seen in Figure 6.10, the lactic acid bacteria counts of the samples coated with protective culture incorporated films increased 4 to 5 log, compared to uncoated samples. During 2 weeks of storage period, there was an inconsiderable slight decrease in the microbial load of beef cubes coated with lactic acid bacteria incorporated alginate films. Thus, it is clear that the *L. delbrueckii* subsp. *lactis* and *L. plantarum* incorporated into alginate films are quite stable at the beef surface. On the other hand, the initial microbial counts of uncoated beef cubes were almost 2 decimal higher than that of uncoated beef cubes. It appeared that the coating of beef cubes with alginate has some protective effect on naturally occurring lactic acid bacteria. However, this effect lasted only for several days and lactic acid counts of coated and uncoated beef cubes reached to the same level during cold storage.

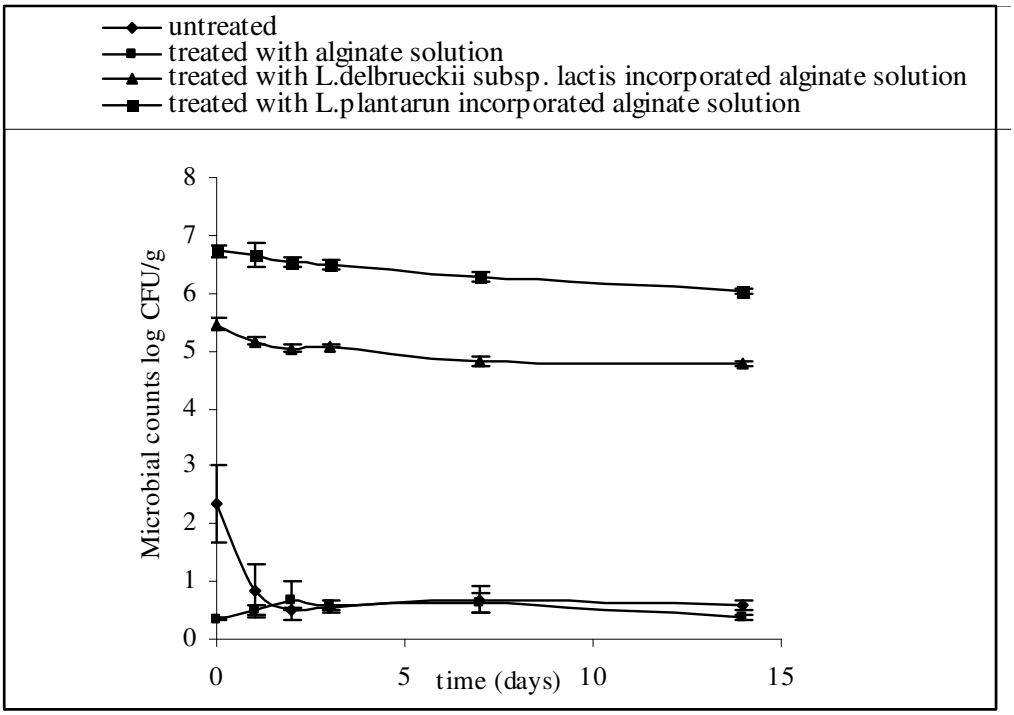


Figure 6.12. The change in the counts of the different lactic acid bacteria incorporated alginate films during storage.

CHAPTER 7

CONCLUSIONS

- The antimicrobial system formed by lactoperoxidase incorporated alginate films, thiocyanate and hydrogen peroxide show antimicrobial activity on different Gram (+) and Gram (-) bacteria. The system also showed sufficient antimicrobial activity in food application conducted on refrigerated fresh calamari.

- The duration of antimicrobial effect of lactoperoxidase system depends on kinetic properties of the enzyme, initial concentrations of hydrogen peroxide and thiocyanate, and catalase activity of the food product. Thus, it is essential to use suitable concentrations of each ingredient during food applications and choose food with low catalase activity at the surface.

- For food applications, dry powders of thiocyanate and lactoperoxidase preparation can be mixed with alginate powder. Once this mixture is solubilized in water, the foods can be coated with alginate and further treated with $CaCl_2$ to cross-link the film. The hydrogen peroxide can be employed before transportation or storage.

- The lactic acid bacteria can also be incorporated into alginate films. The bacteria showed sufficient stability in the films and on surface of refrigerated beef cubes.

- In industry, the lactic acid bacteria incorporated films can be used in coating of refrigerated fresh and processed meat products to provide a protective effect in case of temperature abuse.

- For food applications, dry powders of lactic acid bacteria can be mixed with alginate powder. Once this mixture is solubilized in water the foods can be coated with alginate and further treated with $CaCl_2$ to cross-link the film. The films can be employed before transportation to market.

- Further studies are needed to test the effectiveness of the developed films on different foods during refrigerated storage and temperature abuse.

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APPENDICES

APPENDIX A

E. coli

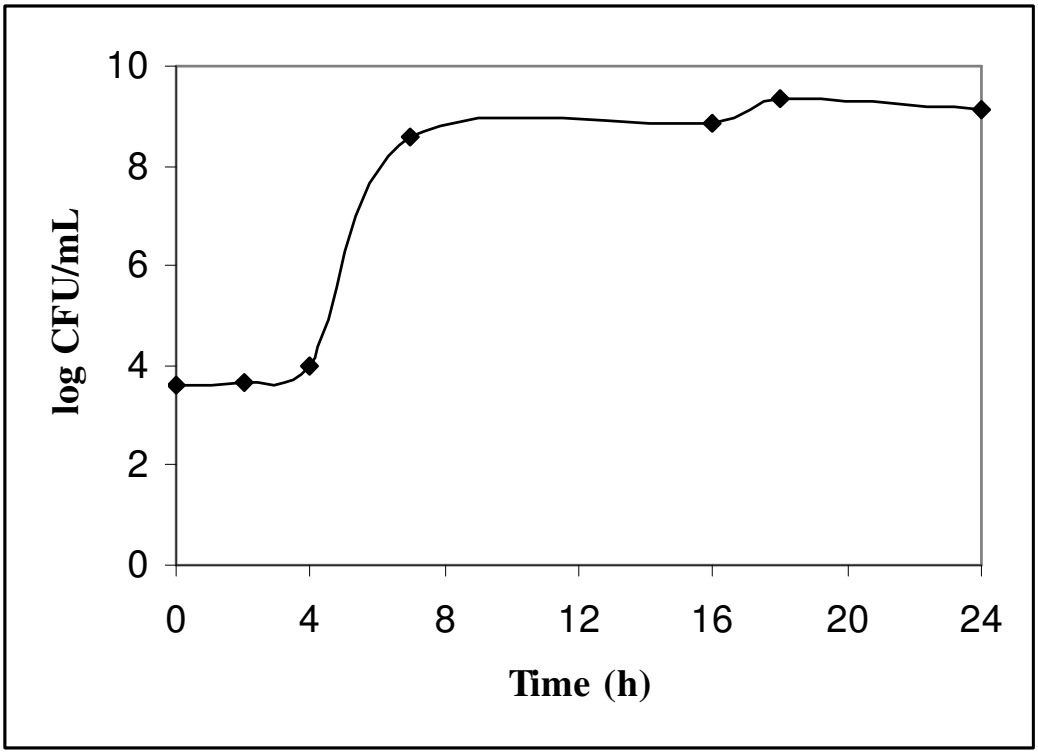

Figure A.1. The growth curve of *E. coli*

APPENDIX B

L. innocua

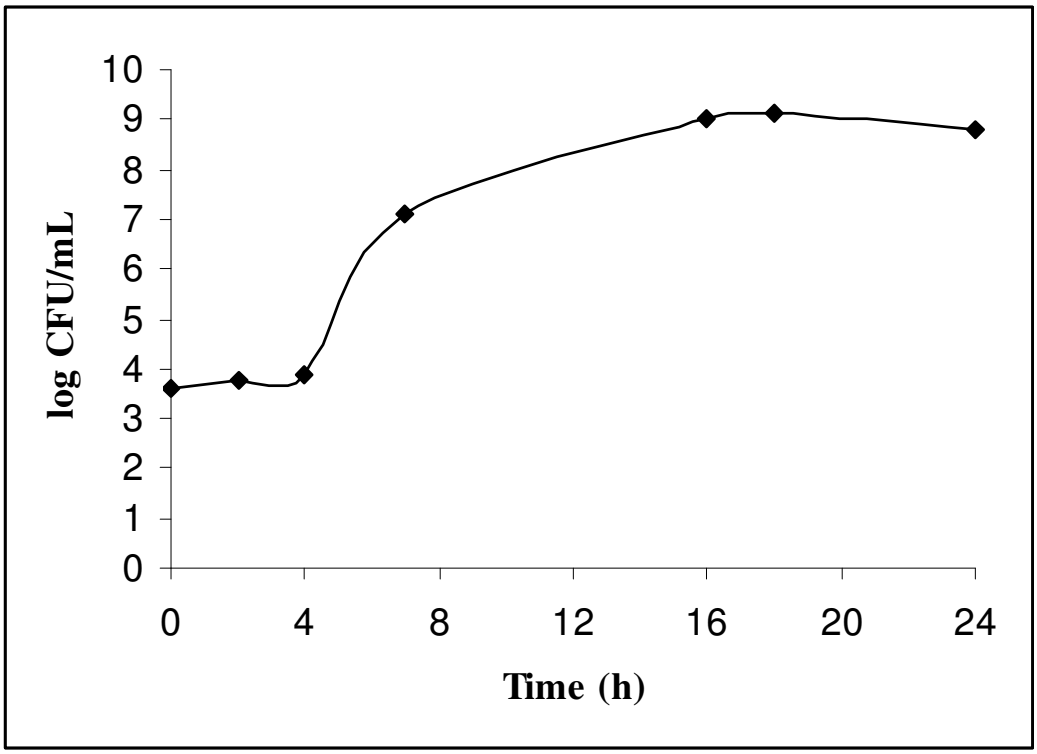

Figure B.1. The growth curve of *L. innocua*

APPENDIX C

P. fluorescens

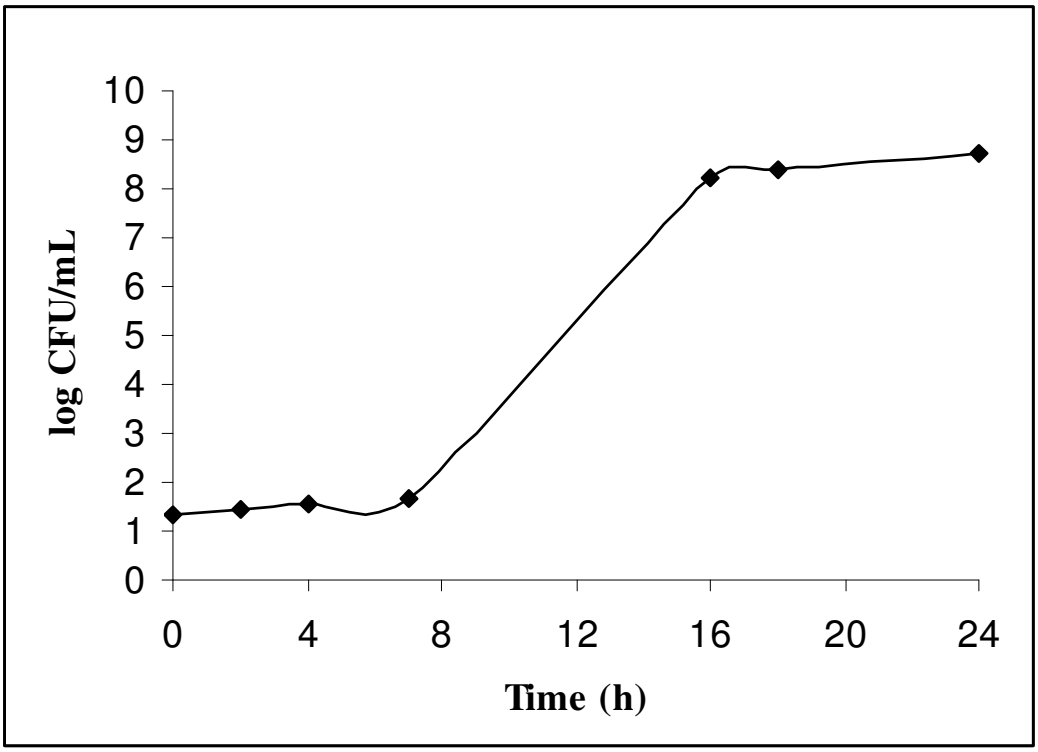

Figure C.1. The growth curve of *P. fluorescens*