PHYSICAL AND ANTIMICROBIAL PROPERTIES OF BIOFILMS CONTAINING NATURAL ANTIMICROBIAL AGENTS

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ABSTRACT

PHYSICAL AND ANTIMICROBIAL PROPERTIES OF BIOFILMS CONTAINING NATURAL ANTIMICROBIAL AGENTS

The goal of this study is to evaluate physical and antimicrobial properties of biopolymers prepared from gluten (WG) and methyl cellulose (MC) with incorporation of activated lactoferrin (ALF), rosemary extract (RE) and natamycin (NA). The effectiveness of agents and antimicrobial films were investigated by agar disc diffusion method. Mechanical and water vapor permeability (WVP) of antimicrobial films were also examined. Film structure was investigated by scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR). Application of films was studied on Kashar cheese inoculated with target molds during 30 days storage at 10^{o}C.

ALF and RE did not show inhibitory activity against tested microorganisms. Minimum inhibitory concentration (MIC) of NA was found 750 ppm against *Aspergillus niger* and *Penicillium roquefortii*. Both films containing NA showed antifungal activity concentrations at and above 2 and 1 mg NA/10 g film solution (fs) against *A. niger* and *P. roquefortii*, respectively. RE in combination with NA reduced MIC of NA against *A. niger* to 1.5 mg NA/10g fs in both films. FTIR spectroscopy did not indicate any interaction between NA and both films. SEM observations showed that NA crystallizes at high concentrations in biopolymers. There was no significant change in WVP of films containing NA. Incorporation of NA to WG film did not cause major changes in its mechanical properties. Reduction in tensile strength of MC films was observed at high NA concentration. Application studies demonstrated the efficacy of WG and MC films including NA against *A. niger* and *P.roquefortii* inoculated on Kashar cheese.

ÖZET

DOĞAL ANTİMİKROBİYAL MADDE İÇEREN BİYOFİLMLERİN FİZİKSEL VE ANTİMİKROBİYAL ÖZELLİKLERİ

Bu çalışmanın amacı, aktif laktoferrin (ALF), biberiye özütü (RE) ve natamycin (NA) içeren buğday proteini (WG) ve metil sellülozdan (MC) elde edilmiş biyopolimerlerin antimikrobiyal ve fiziksel özelliklerinin incelenmesidir. Doğal maddelerin ve bu maddeleri içeren filmlerin antimikrobiyal özellikleri disk difüzyon metodu ile belirlenmiştir. Ayrıca antimikrobiyal aktivite gösteren filmlerin su buharı geçirgenliği ve mekanik özellikleri belirlenmiştir. Fimlerin yapısal özellikleri taramalı elektron mikroskobu (SEM) ve orta bölge kızıl ötesi spektrası (FTIR) analizleri ile incelenmiştir. Son olarak, antimikrobiyal filmlerin, *Aspergillus niger* ve *Penicillium roquefortii* ile kontamine edilen kaşar peynirleri üzerinde paketleme malzemesi olarak kullanımı 30 gün süreyle buzdolabı sıcaklığında incelenmiştir.

Test edilen maddelerden ALF ve RE kullanılan mikroorganizmalara karşıherhangi bir antimikrobiyal etki göstermezken NA'nin *A. niger* ve *P. roquefortii'* ye karşı en düşük etki eden konsantrasyonu 750 ppm olarak bulunmuştur. NA içeren WG ve MC filmlerin aynı funguslara karşı etki gösterdikleri en düşük konsantrasyonlar sırasıyla 2 ve 1 mg NA/10gr film solüsyonu (fs)'dur. RE tek başına antifungal özellik göstermezken NA ile birlikte filmlerin yapısına katıldığında *A. niger*'e karşı sinerjistik aktivite göstermiş ve NA'nin her iki film içindeki en düşük etki eden konsantrasyonunu 1.5 mg NA/10gr fs'nuna düşürmüştür. FTIR analizleri NA ile filmler arasında herhangi bir etkileşim olmadığına işaret etmektedir. SEM fotoğrafları yüksek konsantrasyonda NA'nin filmler içinde kristalize olduğunu göstermektedir. NA eklenmesi filmlerin su buharı geçirgenliğinde önemli bir değişikliğe neden olmamıştır. NA ilave edilmesi WG filmlerin mekanik özelliklerinde değişikliğe neden olmazken MC filmlerin ise gerilme mukavemetleri yüksek NA konsantrasyonunda düşüş göstermiştir. Kaşar peynirleri üzerine denenen NA içeren WG ve MC filmler etkili sonuç sergilemiştir.

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CHAPTER 1

INTRODUCTION

Food quality and safety are the major concerns for the food industry and have received great attention due to increasing consumer demands for minimally processed and fresh foods. However, microbial growth on the food surfaces originating from post-processing contamination during handling and packaging leads to food spoilage, serious illnesses and consequent economic losses, especially in the presence of food borne pathogenic bacteria and molds (Eswaranandam, et al. 2004).

Antimicrobial agents could be used onto the food surfaces via direct application techniques such as dipping, spraying or brushing to control undesirable microorganisms in foods during storage and distribution. However, direct application of antimicrobial substances may result in neutralization or evaporation of active agents and rapid diffusion into the bulk of the foods. Incorporation of natural antimicrobial agents into the biopolymers also known as edible films is an innovative way of overcoming those problems. Use of antimicrobial biopolymeric packaging materials could provide not only the extension of the shelf life of the food products but also elimination of environmental concerns due to utilization of synthetic polymers (Coma, et al. 2002, Kristo, et al. 2007).

Biopolymers obtained from proteins and polysaccharides have been manipulated to prepare packaging materials which could have favorable barrier properties against moisture, oxygen, aromas and also could function as carriers for antimicrobial agents (Ustunol and Mert 2004, Sebtı, et al. 2005). Films prepared from wheat gluten (WG) are extensively studied protein based polymers. WG has easy film forming properties, renewable and biodegradable nature. It also shows excellent oxygen and carbon dioxide resistance at low relative humidities (Roy, et al. 1999, Micard, et al. 2000). Cellulose, major structural component of plants, is one of the most abundant renewable resources and its derivatives have excellent film forming properties. Methyl cellulose (MC) is formed by the alkali treatment of cellulose followed by reaction with methyl chloride. MC films have flexible and transparent character. They also posses low oxygen and

moisture vapor transmission rates when compared to other hydrophilic edible films (Peressini, et al. 2003, Ruckenstein and Park 2001, Debeaufort and Voilley 1997).

Incorporation of biologically derived antimicrobial materials into the edible films has received interest of the food industry as active packaging materials to eliminate the surface contamination of products. Examples of natural agents added to biopolymers include bacteriocins such as nisin and pediocin, enzymes like lysozyme and extracts from natural sources (Padgett, et al. 2000, Cutter, et al. 2001, Ozdemir and Floros 2001).

Antimicrobial edible films also must maintain adequate physical and barrier function that affect the usefulness of the films during distribution and storage. Thus, combined analysis of antimicrobial, tensile, barrier and physical properties are important to predict the behavior of edible films in food systems (Min, et al. 2005).

In this study, biopolymers based on WG and MC by incorporation of natural antimicrobial agents, namely activated lactoferrin (ALF), rosemary extract (RE) and natamycin (NA) were produced for food packaging applications. The effectiveness of natural agents both directly in solid medium and added into film solution was evaluated against three important food borne pathogenic bacteria: *Listeria monocytogenes* (*L .monocytogenes*), *Escherichia coli* O157:H7 (*E.coli* O157:H7), *Salmonella enteritidis* (*S. enteritidis*) and two spoilage molds: *Aspergillus niger (A. niger)* and *Penicillium roquefortii (P. roquefortii).* Effective antimicrobial releasing films were examined in terms of their mechanical and barrier (water vapor) properties as well as the interaction between active agent and the polymer. Finally, application of the films on real food system was also investigated at refrigeration temperature during 30 days storage.

This thesis consists of six chapters that mainly focus on preparation, characterization and application of antimicrobial WG and MC films. In Chapter 1, basic introduction of the topic and goals of this study are provided. Chapter 2 gives the detailed information about food borne pathogenic bacteria and molds causing food borne illnesses. Natural antimicrobial agents for the preservation of foods are also outlined in this part. Properties of biodegradable packaging materials especially details of WG and MC films and their application in food industry together with literature survey are presented in Chapter 3. Materials, preparation of films, antimicrobial testing methods of agents, characterization of effective films with regard to antimicrobial, mechanical, barrier and physical properties are explained in Chapter 4. This chapter also describes application methods of films on Kashar cheese samples. The results of the

study are presented and discussed in Chapter 5. Finally in Chapter 6, joined conclusion and future needs of potential WG and MC films incorporated with natural antimicrobial agents for the food application are stated.

CHAPTER 2

NATURAL ANTIMICROBIAL AGENTS FOR THE PRESERVATION OF FOODS

Foods can deteriorate in quality during distribution, processing and storage due to the chemical reactions, physical changes or the presence and the growth of the microorganisms on the food surfaces (Lee, et al. 2004). Post process contamination of foods could originate from mishandling of the product and faulty packaging. It is one of the major factors causing food spoilage and increase in the risk of food borne diseases (Kristo, et al. 2006). Contamination of foods by undesirable microorganisms such as mold, yeasts and bacteria poses great impact on the shelf life and sensory properties of the foods and also causes significant health and economic concerns.

Recontamination of ready-to-eat (RTE) meat and meat products by the food borne pathogenic bacteria such as *L. monocytogenes*, *S.typhimurium, S.enteritidis, E.coli* O157:H7, *Yersinia enterolitica* and *Pseudomonas* sp is considerable concern for consumers and food producers. Approximately 76 million cases of food borne illnesses, 325000 hospitalizations and 5200 deaths have been reported in the USA every year due to consumption of meat products (Mead, et al. 1999). According to statistics originated from food borne diseases in Turkey, nearly 7875 hospitalizations and 324 deaths were reported in 2002 (Saglık 2002).

L. monocytogenes is recognized as one of the most important food borne pathogen since it can survive for a long period of time under severe conditions and can grow at temperatures as low as 2^{o}C to 4^{o}C (Min, et al. 2005). Therefore, refrigerated storage is not an effective way to prevent the growth of this microorganism (Lungu, et al. 2005). Listeriosis has been associated with consumption of foodstuffs such as milk, soft cheese, fresh and frozen meats and sea foods. It was reported that approximately 2500 illnesses and 500 deaths occur because of human listeriosis caused by Listeria every year in USA and 2298 cases of food borne listeriosis resulted in nearlly $2.33 billion (about $1 million per case) economic losses in USA in 2000 (Min, et al. 2005, Cagri, et al. 2002).

E.coli O157:H7 is also responsible for many outbreaks related to foods including ground beef (Bell, et al. 1994) and fermented meat product (Tilden, et al. 1996). In 2000, 62458 infections were reported and the estimated annual cost of these cases was $659.1 million (about $10500 per case) in USA (Cagri, et al. 2001).

Salmonellosis caused by contamination of foods such as meat, poultry and dairy products by *Salmonella sp* is another significant threat to human health and also to the food industry. In USA, annually 2 to 4 million cases of salmonellosis occur and it was estimated that the combination of medical costs and lost productivity was 2.4 billion dollars in 2000 (Min, et al. 2005).

In addition to the growth of pathogenic bacteria on the food surface, mold contamination also causes considerable economic losses for the dairy producers and poses significant health risk for people due to the production of mycotoxins. Systematic distribution of molds from Turkish mycoflora is illustrated in Figure 2.1.(Topal 2004).

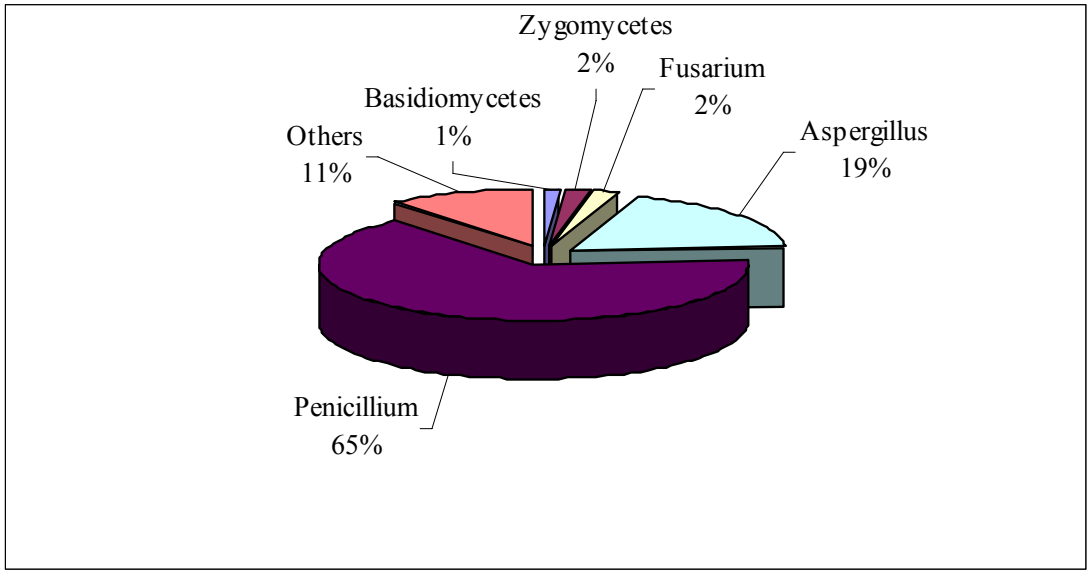

Figure 2.1. Systematic distributions of molds present on foods in Turkey (Source: Topal 2004)

Aspergillus niger is commonly found in food contamination cases and causes food spoilage especially in dairy products. It is also an opportunistic human pathogen which leads to pulmonary disease in immuno-compromised patients. Moreover, *A. niger* causes changes in the organoleptic properties food products such as cheese, nuts and oilseeds by producing secondary metabolites (Sebti, et al. 2005). *P. roquefortii* is used

in the manufacturing of Roquefort cheese and other varieties of blue cheeses. On the other hand, it is also described as a major fungal contaminant which affects the quality characteristics of some cheeses such as Jarlsberg, Norvegia, Kashar and Cheddar cheeses (Kure, et al. 2001). Furthermore, Erdogan et al. (2003) found that *P. roquefortii* isolates from Tulum cheese produced some mycotoxins containing patulin, penicillic acid, roquefortine or PR toxin.

Development of new processing methods and production of new antimicrobial agents to produce safer foods have been a priority research area for scientists working on foods. Several chemicals such as organic acids and their salts, alcohols, ammonium compounds or amines and sulfites are commonly used as antimicrobial agents to eliminate the surface contamination of foods (Appendini and Hotchkiss 2002). However, there has been an increasing concern of using chemical agents among consumers because these preservatives could be toxic to human. In addition, microorganisms may become resistant to these agents due to their uncontrolled use (Souza, et al. 2005). Natural antimicrobial agents obtained from natural sources such as microorganisms, milk and plants could be alternatives for chemical additives.

2.1. Antimicrobial Agents Derived from Milk

Milk has antimicrobial fractions such as immunoglobulins, antibodies, lactoferrin, lactoperoxidase and lysozyme. Also casein, fatty acids and volatiles found in milk pose antimicrobial properties. Especially, lactoferrin, lactoperoxidase and lysozyme are commonly used for the preservation of foods.

2.1.1. Lactoferrin

Lactoferrin (LF) was first isolated from milk in the late 1950s and defined as an iron-binding glycoprotein which is similar to those of transferrin family. It is a single-chain polypeptide with the molecular weight of 80-kDa. This positively charged protein consists of two symmetric N and C globular lobes that are connected by a “hinge region”. Each lobe is capable of binding one metal ion such as Fe^{2+} , Fe^{3+} , Cu^{2+} , Zn^{2+} (Anderson, et al. 1989). LF is also found in other exocrine fluids such as saliva, tears and animal tissues (Diarra, et al. 2002). Chemical structure of LF is displayed in Figure 2.2.

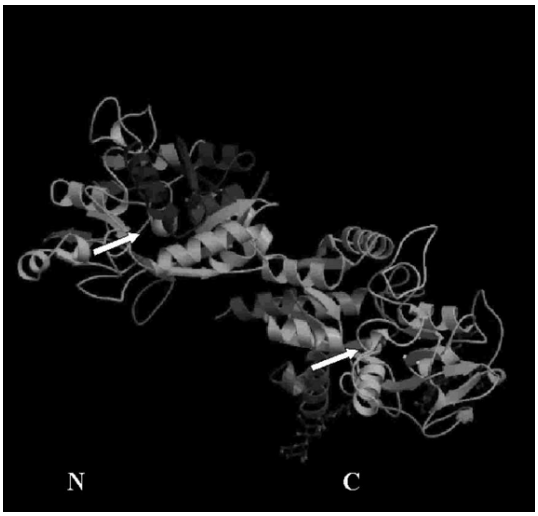

Figure 2.2. Chemical structure of LF. The N-lobe (N) and C-lobe (C) are bound to each other by the hinge. Fe-ions is represented by arrows (Source: Anderson, et al. 1989)

Concentration of LF in milk varies depending on the species. Although human (0.2-0.5g/100 ml), pig and mice milk contain high concentration of LF, level of LF in cow and other ruminants is low (Davidson and Branen 1993).

Antimicrobial activity of LF has been reported against several bacteria such as *Staphylococcus aureus, Klebsiella sp*, and *E. coli* (Davidson and Branen 1993). LF acts on bacteria by binding the iron in the environment. Iron is an essential element required for the growth of the bacteria (Min, et al. 2005). Besides this bacteriostatic action, LF exerts bactericidal activity by damaging the outer membrane of gram negative bacteria and causing a rapid release of lipopolysaccharides with an associated increase in membrane permeability (Orsi 2004). In addition, antiviral activity of LF have been demonstrated against many enveloped and naked viruses such as herpes simplex virus1 and 2 (Hasegawa, et al. 1994), hepatitis C virus (Ikeda, et al. 2000) and poliovirus (Marchetti, et al. 1999). LF prevents entry of virus in the host cell via affecting cellular receptors or binding to the virus particles itself (Strate, et al. 2001). Regarding the antifungal activity, LF is effective on some pathogens like *Candida* and *Trichophyton* cells. It causes iron deprivation and damage to the cell wall of fungi (Orsi 2004).

Wakabayashi et al. (2006) discovered an antimicrobial peptide known as lactoferricin which was obtained by pepsin digestion of bovine lactoferrin. It was demonstrated that it has greater antimicrobial action than LF. The mode of action of

lactoferricin has not been elucidated but it is believed that this peptide affects the surface of bacteria because of its high number of basic amino acid residues (Wakabayashi, et al. 2006).

2.1.2. Activated Lactoferrin

Although LF isolated from milk exerts good antimicrobial activities against several bacteria, molds and viruses, it is highly susceptible to pH changes, heat and proteolysis. Activated lactoferrin (ALF) that is an immobilized form of commercial lactoferrin has been developed to enhance its antimicrobial properties. This natural compound acts as a microbial blocking agent which prevents binding of microorganisms to biological surfaces or tissues and inhibits the microbial growth by several mechanisms (Naidu 2002). Bacteria can easily bind to the tissue components such as collagen which provides anchor sites for attachment. ALF also binds to this site with higher affinity than bacteria therefore blocking tissues interaction with bacteria. ALF has also ability to bind to iron, microbial cell surfaces and inhibits the formation of fibria which is the effective colonization factor in bacteria (Locke, et al. 2003). Damaging of outer membrane of gram negative bacteria as the bactericidal action mechanism of ALF has been reported (Naidu 2003). ALF showed a potent antimicrobial action against several food borne pathogens such as *E. coli* O157:H7*, L. monocytogenes, Salmonella sp* and food spoilers including *Bacillus sp.* ALF is considered as Generally Recognized as Safe (GRAS) substance (CFR 170.36) by the Food and Drug Administration (FDA) in USA. It is permitted at levels of 65.2 mg/kg of beef (Naidu 2002).

2.1.3. Lactoperoxidase

Lactoperoxidase (LP) is an antimicrobial system found in milk, saliva and other body fluids (Jacob, et al. 2000). It consists of three components; the LP enzyme, an oxidizable substrates and hydrogen peroxide (H_2O_2). All of these components have to be present in the system for the antimicrobial effect (Davidson and Branen 1993).

LP is an abundant enzyme in cow and mammalian milk. Concentration of LP in milk could be affected by the diet, breeding and some hormones such as estrogen. Thiocycanate, idican (indoxyl sulfuric acid), bromide iodide and other halides can be

used as an oxidizable substrate (electron donor) for the LP system. In milk, thiocycanate, which is a ubiquitous anion in animals, serves as the primary substrate and it is generally present in milk up to 15 μg/ml. H_2O_2 is another essential component of LP system. Although milk does not have H_2O_2, microbial metabolism can produce this agent under aerobic conditions to activate the system (Davidson and Branen 1993).

LP enzyme catalyzes the oxidation of thiocyanate (SCN^-) by H_2O_2 to hypothiocyanous acid (HOSCN), hypothiocyanate ($OSCN^-$) and other short lived antimicrobial peptides. These agents react with sulfhydryl groups of enzymes and affect glycolytic pathways, amino acid transport system and oxidation of cytoplasmic membranes (Min, et al. 2005, Touch, et al. 2004). LP system is active against several bacteria, fungi, viruses and parasites (Min and Krochta 2005, Jacob, et al. 2000, Pakhanen and Aalto 1997, Seifu, et al. 2005).

2.1.4. Lysozyme

Natural systems such as milk, eggs and tears contain lysozyme which is a lytic enzyme and described as *N*-acetylhexosaminidase. Hen egg white includes 2250-3270 μg/ml of lysozyme, while bovine milk has nearly 13 μg lysozyme/100 ml milk. Thus, hen egg is the major source of this enzyme (Davidson and Branen 1993).

It inhibits the bacteria by hydrolyzing the β (1-4) linkages between *N*-acetylmuramic acid and *N*- acetylglucosamine of the peptidoglycan layer of bacterial cell wall. It is more effective against grampositive bacteria than gram negative ones since lipoprotein-lipopolysaccharide layer in gram negative bacteria prevent the access of lysozyme to the cell wall. However, some chemicals such as ethylenediaminetetracetic acid (EDTA) act synergistically with lysozyme and enhance its action against gram negative bacteria (Davidson and Branen 1993).

2.2. Antimicrobial Agents Produced by Microorganisms

2.2.1. Natamycin

Natamycin (NA) produced by fermentation using *Streptomyces natalensis* is a novel highly active antibiotic. Pimaricin, synonyms of natamycin, is widely used to retard the mold and yeast growth in the food industry (Stark 2003).

Utilization of NA on the surface of the foods has gained much attention owing to its unique characteristics. It is safe for the consumer and effective against several molds and yeasts at low concentration. NA also does not affect the quality of foods when it is applied on the food products like cheese and sausage. It has no activity against bacteria therefore bacterial ripening process of foods such as cheese is not influenced in negative way (Stark 2003).

2.2.1.1. Physical and Chemical Properties

Natamycin is a polyene macrolide antibiotic which is characterized by its carbon-carbon double bonds. It has the empirical formula of C_{33} H_{47} NO_{13} with the molecular weight of 665.7. Natamycin has amphoteric character and its isoelectric point is 6.5. It is extremely insoluble in water, higher alcohols, esters, ketones and various oils because of its large molecular weight and conjugated double bond structure. Glycerol, propylene glycol and acetic acid are the best solvents for natamycin. It was also reported that at high or low pH values, the solubility of natamycin is increased. It is a white odorless and tasteless powder and can be stored for many years at room temperature in the absence of moisture and light. Gamma irradiation and ultraviolet light with wavelengths of 300-350 nm inactivates this antibiotic (Brik 1981, Stark 2003). Chemical structure of natamycin is shown in Figure 2.3.

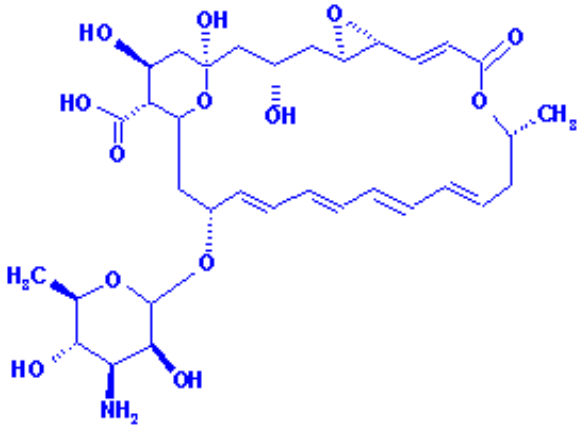

Figure 2.3. Chemical structure of natamycin

(Source: Wikipedia 2008)

2.2.1.2. Mechanism of Action

Lipids, proteins, phospholipids and sterols are the main components of eukaryotic cell membrane. Like the other polyene antibiotics, natamycin binds to the ergestrol which is the major sterol in the cell membrane of molds and yeasts. This damages permeability of the cell membrane. Increased permeability leads to leakage of essential elements such as K^+, NH_4, inorganic phosphate, nucleotides and causes cell lysis. Since bacteria and mammalian cell do not contain ergesterol in their cell membrane, natamycin do not show inhibitory effect against these cells (Davidson and Branen 1993).

2.2.1.3. Applications

Natamycin is commonly used in many countries as food additive to protect food products from mold and yeast. It has been approved as GRAS in the United States (CFR 172.155) by FDA (Stark 2003). Due to its low solubility, natamycin remains on the surface of the product and hardly penetrates into the food, thus this makes natamycin suitable for use as a surface treatment agent (Davidson and Branen 1993).

Natamycin is applied to several types of cheese such as blue cheese, kashar, Swedish hard cheese, Cheddar and Gongozala cheeses by dipping or spraying in an aqueous solution containing 200-300 ppm. It was also added to the polymer dispersion used as a coating material on cheese (Stark 2003). In addition to cheese samples, sausages are treated with natamycin by dipping (500-1000 ppm) or spraying (2000 ppm) to eliminate fungal growth during ageing and storage (Brik 1981). Several studies reported the antifungal effect of natamycin on Dutch and German sausages and Italian sausages such as dry salami (Davidson and Branen 1993, Stark 2003).

The permitted level of natamycin on the food surface/rinds is $2mg/dm^2$ to a maximum depth of 5mm. However, permitted use of natamycin varies from country to country. In Australia, permitted level of natamycin on cheese sample is 15mg/kg based on individual cheese weight. In USA, the level of natamycin that could be applied on cheese must not exceed 20mg /kg in the final product (Stark 2003).

2.3. Plant Based Antimicrobial Agents

Within the past few years usage of plant extracts as antimicrobial additives in food products has attracted much attention due to increasing consumer demands for foods with out chemical additives (Davidson and Branen 1993). Antimicrobial action of plants, herbs and their derived essential oils and some isolated substances could be attributed to the presence of phenolic compounds, terpenes, aliphatic alcohols, aldehydes, ketones, acids and isoflavonoids in their leaves, flowers, bulbs and fruits. These compounds can completely eliminate the bacteria and fungus or only inhibit the production of their some secondary metabolites such as mycotoxins (Lopez Malo, et al. 1998).

2.3.1 Spices and Essential Oils

Spices are commonly used in food industry for the purpose of enhancing the flavor of foods and safety. Their antimicrobial properties originate from their essential oils which are obtained commercially via distillation processes (Davidson and Branen 1993). Several reports demonstrated inhibitory effect of spices and their constituents on a variety of microorganisms. Examples of plants having antimicrobial activity are displayed in Table 2.1.

Antimicrobial activities of cinnamon, allspice and clove against several food spoilage bacteria, molds and yeasts have been reported (Conner 1983). Eugenol (2-methoxy-4-allyl phenol) and cinnamic aldehyde which are the main volatile oils in these spices are responsible for their antimicrobial actions (Bullerman, et al.1977). Hitokoto et al. (1980) demonstrated the antifungal activities of powdered allspices and ground cloves against mycotogenic molds *A. flavus*, *A. versicolor* and *A. ochraceus* in the growth medium. Oregano, thyme and savory also have antimicrobial activity owing to its volatile compounds, terpenes carvacrol, *p*-cymene and thymol. Allium species such as garlic and onion have also antimicrobial and medicinal properties. Major component obtained from garlic bulbs by steam distillation is allicin or diallylthiosulfinic acid. Allicin is highly pungent and colorless oil that gives the general characteristic, odor and taste of garlic and onion (Davidson and Branen 1993).

Table 2.1. Examples of plants having antimicrobial activity

(Source: Cowan 1999)

Common name	**Scientific name**	**Compound**	**Class**	**Activity**
Allspice	*Pimenta dioica*	Eugenol	Essential oil	Several types of microorganisms
Barberry	*Berberis vulgaris*	Berberine	Alkaloid	Bacteria, protozoa
Basil	*Ocimum basilicum*	Essential oils	Terpenoids	Salmonella, bacteria
Ceylon cinnamon	*Cinnamomum verum*	Essential oils	Terpenoids, tannins	Several types of microorganisms
Clove	*Syzygium aromaticum*	Eugenol	Terpenoid	Several types of microorganisms
Garlic	*Allium sativum*	Allicin,	Sulfoxide	Several types of microorganisms
Olive oil	*Olea europaea*	Hexanal	Aldehyde	Several types of microorganisms
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, Candida
Rosemary	*Rosmarinus officinalis*	Essential oil	Terpenoid	Several types of microorganisms
Savory	*Satureja montana*	Carvacrol	Terpenoid	Several types of microorganisms
Thyme	*Thymus vulgaris*	Affeic acid	Terpenoid	Several types of microorganisms
		Thymol	Phenolic alcohol	Viruses, bacteria, fungi
		Tannins	Polyphenols	
Valerian	*Valeriana officinalis*	Essential oil	Terpenoid	Several types of microorganisms

2.3.1.1. Rosemary Extract

Many of the studies among the herbs and spices have been concentrated on rosemary (*Rosmarinus officinalis* L) because of its antimicrobial and antioxidant properties (Baratta, et al. 1998, Pinto, et al. 2002). Rosemary belonging to the *Labiatae* family is an evergreen perennial woody shrub with aromatic, needle like leaves and gray bark. It is an important medicinal plant and its constituents have a therapeutic potential in treatment of several diseases such as bronchial asthma, spasmogenic disorders and has GRAS statutes (Skandamis, et al. 2001). In addition to its therapeutic application, essential oil of rosemary extract (RE) has strong antioxidant and antimicrobial activity. Several studies investigated the antimicrobial and antioxidant properties of rosemary species and their main constituents (Pandit and Shelef 1994, Basaga, et al.1997, Del Campo, et al. 2000, Celiktas, et al. 2007). Antioxidant and antimicrobial properties of rosemary is attributed to phenolic diterpenes such as carnosic acid, carnosol, rosmanol, epirosmanol, isorosmanol, methyl carnosate and other phenolic acids, such as rosmarinic acid (Cuvelier, et al. 1996, Schwarz and Ternes 1992).

In the study of Baratta et al. (1998) antibacterial activity of commercial rosemary extract was shown against *S.aureus*. Effects of rosemary and its components (α-pinene/1.8-cineole/camphor) against plant parasites, *Neotoxoptera formosana* was also determined (Masatochi, et al. 1997). In spite of its strong antioxidant activity, the rosemary extract obtained by supercritical fluid extraction exhibited low antimicrobial activity against *M. tuberculosis* (Leal, et al. 2003). *R. officinalis* was also found to have a bacteriostatic effect on *S .iniae* (Abutbul, et al. 2004).

2.4 Bacteriocins

Bacteriocins produced by bacteria are ribosomally synthesized and extracellularly released low molecular weight peptides or proteins. They generally contain 30-60 amino acid residues (Settanni and Cosetti 2007). Production of bacteriocins has been encountered in several types of bacteria such as *E. coli*, *Enterecoccus*, *Pediococcus* and *Leuconostoc sp*. In recent years, bacteriocins

originating from lactic acid bacteria (LAB) such as *Lactococcus sp* and *Streptococcus sp* have attracted much more attention due to their GRAS statutes.

Bacteriocins are classified into three groups on the basis of their common and mainly structural characteristics (Cleveland, et al. 2001). Class I bacteriocins also known as lantibiotics are characterized by their unusual amino acid residues like lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Class I can be subdivided into Class Ia and Class Ib according to their antimicrobial activity and chemical structure. Although Class Ia bacteriocins are cationic and hydrophobic, Class Ib are globular and have no net charge or have a net negative charge. Heat –stable, non-lanthionine containing bacteriocins are grouped in Class II. These classes further subdivided into three groups. Class IIa are well known for their antilisterial activity. Class IIb includes bacteriocins which need two different peptides for activity. Class IIc containing *sec* dependent secreted peptides. Heat-labile and >30kDa molecular weight bacteriocins constitute Class III (Cleveland, et al. 2001, Chen and Hoover 2003). Examples of bacteriocins and their activity spectrums are demonstrated in Table 2.2.

Table 2.2. Examples of bacteriocins and their activity spectrums
(Source: Kurt and Zorba 2005)

Bacteriocions	**Producer**	**Activity Spectra**
Class Ia		
Nisin	*Lactococcus lactis*	*Lactococcus sp., Lactobacillus sp., Streptococcus sp., Micrococcus sp., Mycobacterium sp., Staphylococcus aureus, Corynebacterium sp., Clostridium sp., Bacillus sp., Listeria sp.*
Lactocin S	*Lactobacillus sake*	*Lactobacillus sp., Lc.mesenteroides, P. acidilactici, P. pentosaceus*
Epidermin	*Staphylococcus epidermis*	
Gallidermin	*Staphylococcus gallinarum*	
Lacticin 481	*Lactobacillus lactis*	*Lactococcus sp.,L. helveticus, L. bulcaricus,*
Class Ib		
Mersacidin	*Bacillus subtilis*	
Cinnamycin	*Streptomyces cinnamoneus*	
Ancovenin	*Streptomyces sp.*	
Class IIa		
Pediocin PA-1	*Pediococcus acidilactici*	*Lactobacillus sp., Pediococcus sp., L. monocytogenes*
Pediocin AcH	*Pediococcus acidilactici*	*L. monocytogenes, L.innocua*
Sakacin A	*L. sake*	*Lactobacillus sp., L.monocytogenes*
Lactococcin MMFII	*L. lactis*	*Enterococcus sp., Lactobacillus sp., Lactococcus sp., L. ivanovi*
Class IIb		
Lactococcin G	*L. lactis*	
Lactococcin M	*L. lactis*	
Lactacin F	*Lactobacillus johnsonii*	*L. bulcaricus, L. leichmanni, L. helveticus, L. lactis, L. fermentum 1750, E. faecalis*
Plantaricin EF	*L. plantarum*	
Plantaricin JK	*L.plantarum*	
Class IIc		
Enterocin B	*E. faecium*	
Class III		
Helveticin J	*Lactobacillus helveticus*	*L. helveticus, L. bulcaricus*

Since bacteriocins are charge molecules with hydrophobic residue they can easily bind to negatively charged phosphate groups on the target cell membrane via electrostatic interactions. This association results in pore formation and leakage of cellular materials (Cleveland, et al. 2001, Chen and Hoover 2003).

Nisin inhibits the closely related bacteria by forming two types of pore structure. In the "barrel-stave" structure, each nisin molecule binds to the cell membrane perpendicularly and forms an ion channel. In the "wedge" model, nisin binds to phosphate group in the target cell membrane and creates lipid-protein channel (Kurt and Zorba 2005). Though the primary mode of action of nisin is production of pore formation other bacteriocins such as mersacidin inhibits the peptidoglycan synthesis. Colicin has the ability to degrade the DNA and affects the protein synthesis. PediocinPA1 and lactococcin A also lead to voltage independent pores (Cleveland, et al. 2001).

Bacteriocins are applied to food systems by several methods to enhance the safety of foods. They could be used as food additives or foods can be inoculated with LAB which produces bacteriocins. Incorporation of bacteriocins into the packaging materials is an innovative way to control the surface contamination of food products (Chen and Hoover 2003). In the study of Min et al. (1997) nisin and pediocin were applied to casings to inhibit L.monocytogenes in meat and poultry. Scannell et al. (2000) produced bioactive food packaging material using immobilized nisin and lacticin 3147 which reduced the lactic acid bacteria counts in sliced cheese and ham at refrigeration temperatures. To inhibit *S. typhimurium* on fresh broiler skin, nisin was added to polyvinyl chloride, linear low-density polyethylene and packaging materials. Examples of bacteriocins applied to foods are given in Table 2.3.

Table 2.3 Examples of bacteriocins applied to foods
(Source: Cleveland, et al.2001)

Bacteriocin	**Application**	**Result**
Nisin A	Incorporation of nisin into a meat binding system (Fibrimex)	Addition of nisin can reduce undesirable bacteria in restructured meat products
Enterocin 4	Use of an enterocin producer *Ent. faecalis* INIA4 as a starter culture for production of Manchego cheese	Use of an *Ent. faecails* INIA4 starter inhibits *L. monocytogenes* Ohio, but not *L. monocytogenes* Scott A
Linocin M-18	Use of *Bre. lines* as a starter culture for production of red smear cheese	Causes 2 log reduction of *L. ivanovi* and *L. monocytogenes*
Nisin A	Use of nisin to control *L. monocytogenes* in ricotta cheese	Nisin effectively inhibits *L. monocytogenes* for 8 weeks
Piscicolin 126	Use of piscicolin 126 to control *L. monocytogenes* in devilled ham	More effective than commercially available bacteriocins
Leucocin A	Use of a leucocine-producing *Leu. gelidum* UAL187 to control meat spoilage	Inoculation of a vacuum packed beef with the bacteriocin-producer delays the spoilage by *Lactob. sake* for up to 8 weeks
Lactocin 705	Use of lactocin 705 to reduce growth of *L. monocytogenes* in ground beef	Lactocin 705 inhibits growth of *L. monocytogenes* in ground beef

CHAPTER 3

BIOBASED PACKAGING MATERIALS

Food packaging plays an important role in maintaining the food quality and safety by acting as a barrier to light, moisture and microbial spoilage (Weber, et al. 2002). Today, petroleum-derived plastics such as polyolefin, polyethylene, polypropylene and polyamide are commonly used as packaging materials because of their properties like availability in large quantities at low cost, resistance to water vapor, aroma compounds and heat sealability. On the other hand, utilization of synthetic polymers gives rise to environmental problems because they are not biodegradable and derived from non-renewable sources. There is a growing interest in the use of biobased packaging materials as an alternative to synthetic polymers as a result of durability of synthetic polymers with a range of time in nature, limited availability of landfill, some recycling problems and increasing consumer concern over ecological problems (Ko, et al. 2001).

3.1. Biodegradable Polymers

Biopolymer based packaging is a relatively new packaging concept which involves using of raw materials coming from agricultural feed stocks, animal source, marine food processing wastes or microbial sources. Unlike the most synthetic packaging materials, biodegradable polymers are easily broken down to their monomeric unit and finally reduced to O_2, CO_2 ,CH_4 or biomass by means of enzymatic activity of biological systems especially from bacteria and fungi. For that reason, they are not toxic to human and have environmentally friendly nature (Kaplan, et al. 1993). Utilization of biopolymers in food industry as packaging materials has gained much attention due to their structural architecture and diverse chemistry of their side chains providing possibilities to tailor the properties of the final package (Weber, et al. 2002). According to their origin and production method there are three types of biopolymers: 1) extracted directly from natural raw materials, 2) produced by classical chemical

synthesis via renewable bioderived monomers 3) obtained by using microorganisms (Weber, et al. 2002). These three categories are shown in Figure 3.1.

Type 1 polymers are derived commonly from plant and animal sources. Biopolymers prepared from polysaccharides such as cellulose, starch and animal or plant based proteins like wheat, whey and casein are good examples of type I. Although these polymers have excellent barrier properties to gases, their high hydrophilic structure and the excess disulphide cross-linking in some types of protein network lead to problems related to processing and application of high moisture products (Krochta and Johnston 1997, Weber, et al. 2002).

Most commonly available type 2 polymer is polylactic acid (PLA) that has good water vapor barrier properties and low gas transmittance rate. It can be obtained from agricultural sources such as corn, wheat, whey or green juice and wastes (Garde, et al. 2000, Södergaard 2000).

One of the most popular type 3 polymer is poly (hydroxyalkanoate)s (PHAs) produced by many bacterial species in the form of intracellular particles. They are renewable, biodegradable and biocompatible polyesters. In spite of having food packaging properties, production of PHAs is not cost effective (Weber, et al. 2002).

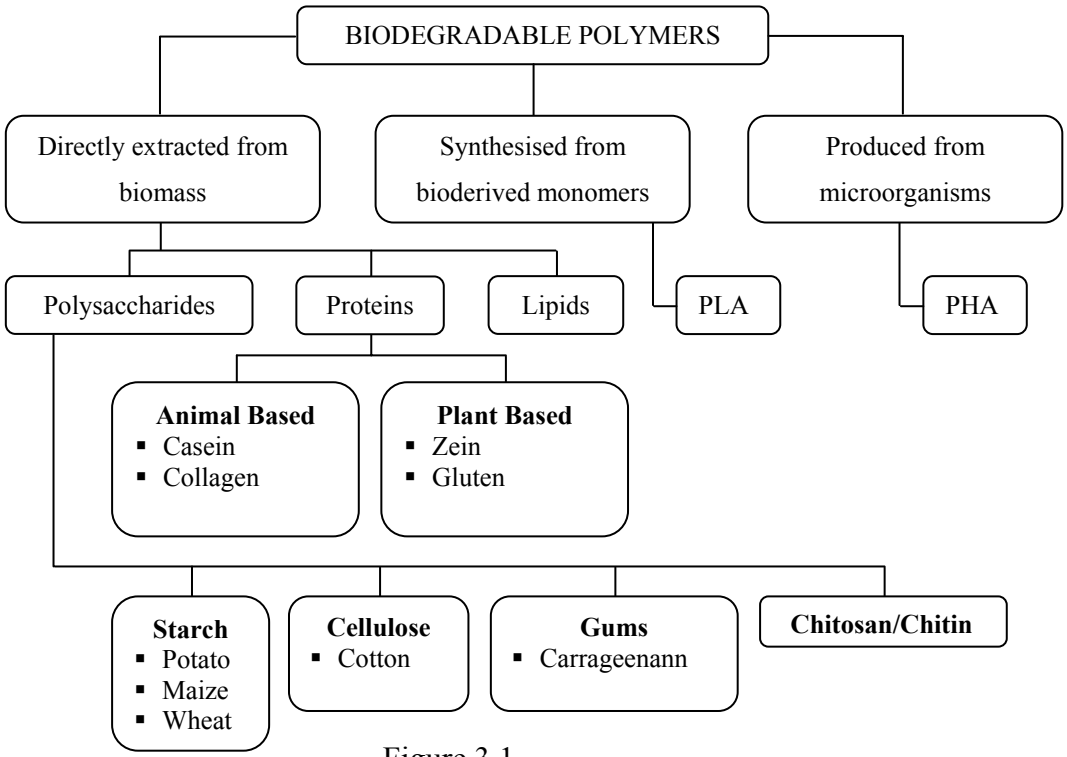

Figure 3.1.

Classification of biobased polymers

(Source: Weber, et al. 2002)

3.2. Biobased Films (Edible Films)

An edible film can be discribed as thin layer of materials produced from biobased polymers (Hernandez-Izquierdo and Krochta 2008). It could be applied directly to food surfaces as coatings or placed between food components as a sheet (Han and Gennadios 2005). Biobased films or coatings might increase the shelf life of the product and improve the quality of food by performing several functions such as acting as a barrier to oxygen, carbon dioxide, moisture, flavor and aroma losses or carrier of antimicrobials, antioxidants (Lacroix and Cooksey 2005, Hernandez-Izquierdo and Krochta 2008). Several functions of edible films and coatings are displayed in Figure 3.2.

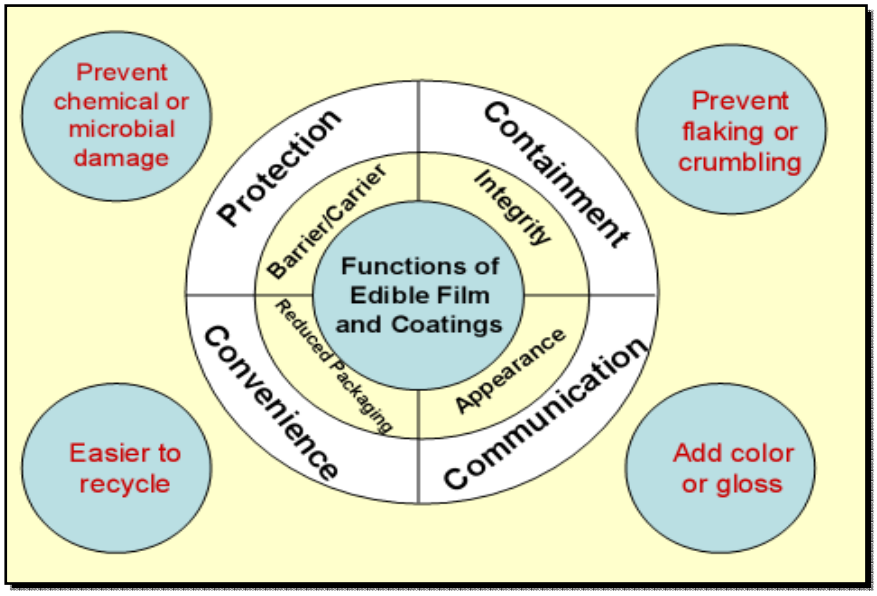

Figure 3.2. Functions of edible films and coatings
(Source: Dangaran, et al. 2000)

Biobased films possess number of advantages over synthetic packaging materials. They could be consumed with food if the composition of film solution is compatible with regulations applied to foods. They could decrease usage of synthetic packaging materials, therefore, contribute to the reduction of environmental pollution. Edible films could also be utilized with non-edible films in a multilayer food packaging.

In that case edible film is designated to contact with food and eliminates the migration of unwanted additives from synthetic films to foods (Robertson 1993, Anker 1996).

One of the main components to produce the edible films is plasticizers. Plasticizers such as glycerol and polyethylene glycol are low molecular weight agents and commonly added to film solution to reduce the brittleness caused by extensive intermolecular forces within the polymer (Krochta and Johnston 1997). Addition of plasticizer into polymer leads to an increase in molecular mobility of the polymer molecules and improves the film flexibility and processability (Guilbert, et al. 1997). However, plasticizers have also adverse effects on the mechanical and barrier properties of the film due to their ability to increase the free volume of the polymer. They also decrease the glass transition temperature which affects the diffusion coefficients for gases or vapor (McHugh and Krochta 1994).

Casting method (solvent process) and dry process (thermoplastic process) are commonly used to produce edible films. Casting method is based on the dispersion of the film solution in food grade solvents and drying of this dispersion (Figure 3.3.). pH, solvent type and drying time are the main parameters that might have an effect on the properties of the films (Guilbert and Gontard 2005). Drying process depends on the thermoplastic properties of biopolymers which is plasticized and heated above their glass transition temperature at low water content by using techniques such as extrusion and injection molding (Guilbert and Gontard 2005). In contrast to casting method, dry process is affected by the mechanical energy input, shear stress level and pressure (Ullsten, et al. 2006).

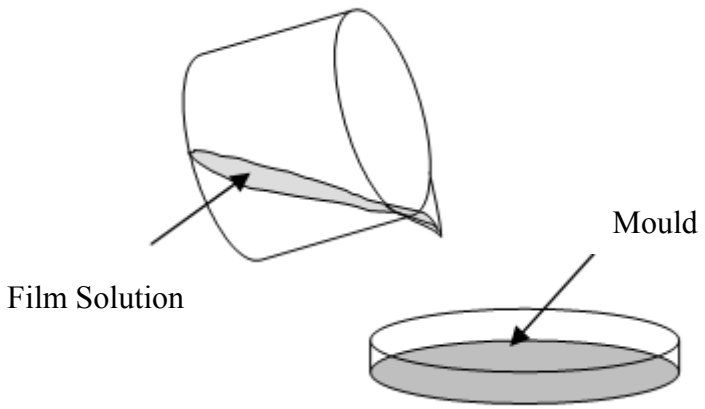

Figure 3.3. Casting method to produce edible films
(Souce: Olabarrieta 2006)

3.3. Types of Biobased Films

Edible films can be obtained from several sources such as polysaccharides, proteins and lipids.

3.3.1. Polysaccharides Based films

Polysaccharides are ideal materials for food packaging applications because they are nontoxic and readily available in nature. Although films formulated from polysaccharides have good barrier to O_2 and CO_2 their water barrier properties are poor due to their hydrophilic structure (Krochta, et al. 1994). Several types of polysaccharides such as starch, cellulose and their derivatives and chitin /chitosan have been tested to prepare films.

3.3.1.1. Starch

Starch is one of the commonly preferred hydrocolloids to form edible films due to its low cost, availability and biodegradability. It consists of (1-4) linked α-D-glucopyranosil units and two distinct polymers, namely amylose and amylopectin. Amylose is the linear fraction of starch and possesses lower molecular size (20-800 kg/mol) (Peressini, et al. 2003). Films based on amylose are coherent, relatively strong and freestanding. Amylopectin fraction is highly branched and has very high molecular weight (5000-30.000 kg/mol). Films obtained from amylopectin are brittle and non-continuous (Chinnan and Cha 2004). In spite of having good barrier to O_2 and CO_2 transmission, starch based films are limited as food packaging materials because of their hydrophilic properties which make them poor barrier to water vapor. Physical and barrier properties of starch films can be improved by blending it with non-biodegradable thermoplastic materials (Arvanitoyannis, et al.1998). Starch-polyethylene, starch–polycrylic acid, starch-polyvinyl alcohol combinations are extensively studied partially degradable packaging films (Kaplan, et al. 1993).

3.3.1.2. Cellulose and Its Derivatives

Cellulose, major structural component of plants, is one of the most abundant renewable resources in nature. It is composed of D-glucose units linked through β-1,4 glycosidic linkage (Figure 3.4.). Native cellulose is insoluble and unswellable in water owing to its highly crystalline structure (Ruckenstein and Park 2001).

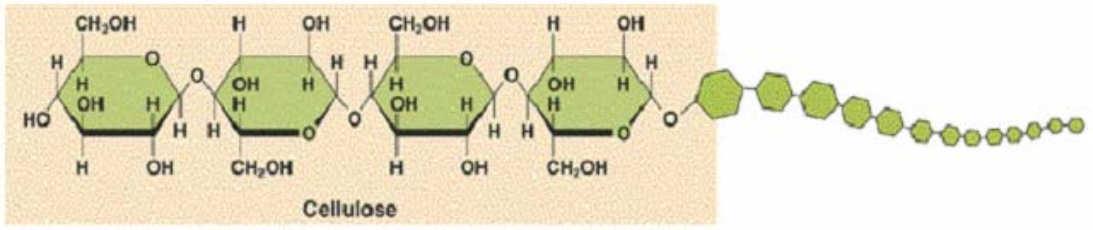

Figure 3.4. Structural formula of cellulose

(Source: Plantphysiology 2007)

Water soluble cellulose derivatives can be formed by partial substitution of hydroxyl groups at positions 2, 3 and 6 on the glucosyl units of cellulose by etherification (Gennadios, et al. 1997). Methyl cellulose (MC) , hydroxypropyl cellulose (HPC) and hydroxypropyl methyl cellulose (HPMC) are water soluble cellulose ethers having ability to produce films. Cellulose based films are transparent, readily peelable and have good barrier properties against O_2, aroma compounds and oil transfer (Erdohan and Turhan 2005).

3.3.1.2.1. Methyl Cellulose Films

MC is a nonionic, water soluble cellulose derivative and produced by reaction of cellulose with methyl chloride under controlled temperatures and pressure (Gennadios, et al. 1997, Krochta, et al. 1994). Chemical structure of MC is shown in Figure 3.5.

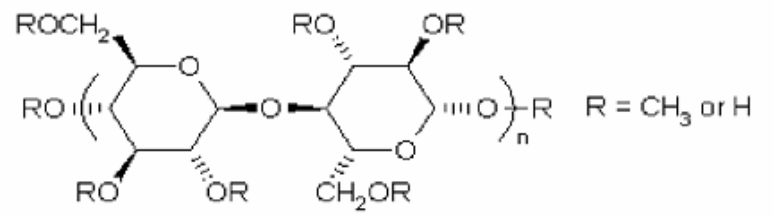

Figure 3.5. Chemical structure of MC

(Source: Sigmaa 2008)

The degree of substitution (DS) that can be defined as the number of substituted hydroxyl groups per anhyrglucose units can vary from 1.6 to 1.9 in commercially produced MC (Erdohan and Turhan 2005). Films based on MC are tough, flexible and transparent due to the linear structure of polymer backbone (Krochta, et al. 1994). MC films are also resistant to oil migration and have low water vapor permeability values compared to other hydrophilic edible films (Erdohan and Turhan 2005).

Several authors have investigated the mechanical and barrier properties of MC films according to film thickness, types of plasticizers, molecular weight and concentration of MC used in film solution. In the study of Park et al. (1993) tensile strength (TS) of MC films was constant with thickness while elongation (E) of the films slightly increased as the thickness increased. Park et al. (1993) also used different types of plasticizers, namely polyethylene glycol (PEG) and glycerin (GLY) to show the effects of plasticizers on mechanical properties of MC films. It was found that generally as the concentration of plasticizers increased E also increased but TS decreased. Among the tested plasticizers, PEG had more effect in terms of increasing flexibility of films than other plasticizer. This result could be attributed to plasticizers properties such as molecular shape, number of carbons and hydroxyl groups. The effect of MC concentration on the mechanical properties of MC based films was also examined (Turhan and Şahbaz 2004). As can be seen from the Table 3.1. when the MC concentration increased both TS and E decreased. Therefore, 3 g/100ml solvent was determined as the most feasible concentration since it has the highest TS and E value among the tested concentrations.

Table 3.1. Effect of MC concentration on the mechanical properties of films (Source: Turhan and Şahbaz 2004)

MC (g/100ml solvent)	TS (MPa)	E (%)
1.5	16±1	10±0.4
3	33±3	14±1
4	23±3	11±1
5	11±1	8±1
6	8±1	6±2

Water vapor permeability (WVP) and oxygen permeability (OP) of MC films were also studied by several researchers (Park, et al. 1993, Turhan and Şahbaz 2004, Erdohan and Turhan 2005). These studies revealed that as the molecular weight (MW) of MC increased both WVP and OP increased (Table 3.2.).

Table 3.2. Changes in oxygen and water vapor permeability of MC with molecular weight (Source: Park, et al. 1993)

Films (MW)	Oxygen Permeability (fl.m/m.s.Pa)	Water Vapor Permeability (ng.m/ m.s.Pa)
13.000	3.1 ± 0.30	0.084 ± 0.0047
20.000	3.6 ± 0.56	0.094 ± 0.0056
41.000	4.6 ± 0.31	0.103 ± 0.0099
63.000	5.3 ± 0.72	0.110 ± 0.0065
86.000	5.1 ± 0.59	0.121 ± 0.0152

3.3.1.2.2. Hydroxypropyl Cellulose (HPC)

HPC, later named as nature-seal, is obtained by the treatment of cellulose with propylene oxide in alkaline environment. It is soluble in cold water and in many hot or cold polar organic solvents. One of the most crucial properties of HPC is its water soluble thermoplastic behavior which allows the use of dry processes such as extrusion or injection molding (Krochta, et al. 1994). The structural formula of HPC is demonstrated in Figure 3.6.

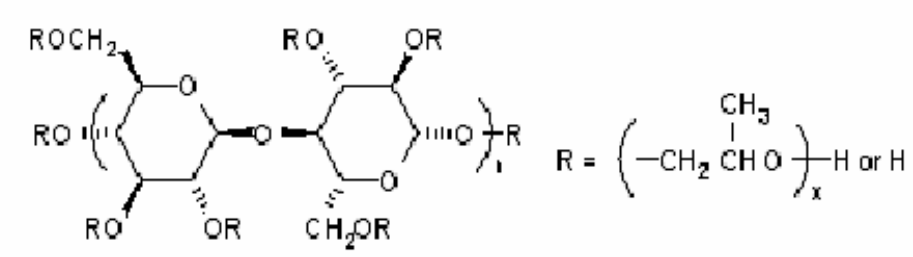

Figure 3.6. The structural formula of HPC
(Source: Sigmaa 2008)

HPC based films or coatings are used to decrease the moisture and oil migration in fried foods and to prevent the lipid oxidation for snacks such as roasted peanuts (Weber, et al. 2000).

3.3.1.2.3. Hydroxypropyl Methyl Cellulose (HPMC)

HPMC is a kind of cellulose ether which is formed by reaction of alkaline cellulose with methyl chloride and propylene oxide. Due to the presence of hydroxypropyl groups HPMC is highly soluble in water and organic solvents (Krochta, et al. 1994). It is used in food industry as an emulsifier, thickening agent, and stabilizer, film former, protective colloid and fat barrier in food products like ice cream, breading and bakery goods (Sigmaa 2008). Chemical structure of HPMC can be seen in Figure 3.7.

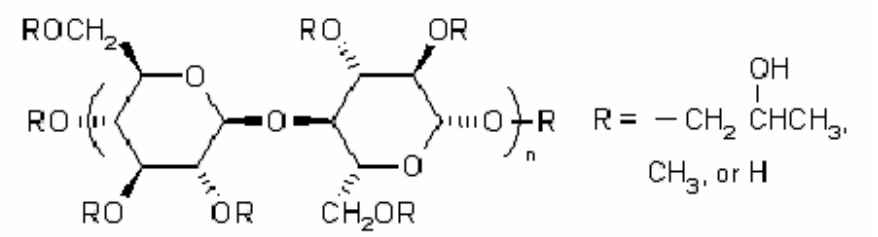

Figure 3.7. The structural formula of HPMC
(Source: Sigmaa 2008)

3.3.1.3. Chitosan Based Films

Chitosan, mainly composed of β (1-4) 2-amino-2 deoxy-D-glucose units, is a linear binary heteropolysaccharide prepared by deacetylation of chitin (Sebastien, et al. 2006). It is the second most abundant natural biopolymer after cellulose (No, et al. 2007) and commonly present in living organisms such as shellfish, insects and mushrooms. Chitosan has been shown to be nontoxic, biodegradable and biocompatible polymer and is used in food industry as film or coating on variety of foods (Sebastien, et al. 2006).

Antimicrobial properties of chitosan based edible films have been revealed against several bacteria such as *L. monocytogenes* and *L. innocua* (Coma, et al. 2002). The mechanism of the antibacterial activity of chitosan could be attributed to interactions between positively charged chitosan molecules and the negatively charged microbial cell membrane thus resulting in leakage of proteinaceous molecules and other intracellular components (No, et al. 2007). Antifungal properties of chitosan based films have been also demonstrated against mycotoxinogen fungal strains (Sebastien, et al. 2006).

3.3.2. Protein Based Films

Several types of proteins originated from animal and plant sources have been used to produce edible films or coatings. When compared to other film forming materials proteins have significant characters such as conformational denaturation and electrostatic charges that make them good raw materials for developing films (Han and Gennadios 2005). Protein based films exhibit good O_2, CO_2 and lipid barrier properties at low relative humidity but their hydrophilic character make them poor barrier to water vapor (Lacroix and Cooksey 2005). Proteins derived from plants include wheat gluten, corn zein and soy proteins. Animal sources like whey proteins, casein and collagen are also utilized commonly to produce films.

3.3.2.1 Wheat Gluten

Gluten, the main storage protein in wheat, is obtained after starch is washed away from wheat flour dough. Commercially, wheat gluten (WG) is a relatively cheap (approximately $1/kg) industrial by product of wheat starch production with wet milling. WG is composed of storage proteins (70-80% dry matter basis), lipids (6-8%), starch and non starch polysaccharides (10-14%) and minerals (0.8-1.4%). Among the other plant proteins WG has unique properties such as good adhesiveness, cohesiveness and film forming abilities once plasticized (Gennadios 2002, Lens, et al. 2003). Gluten is a protein mixture consisting of albumins, globulins, gliadins and glutenins. Albumins and globulins are water and salt soluble, respectively and account for 15-22% of total

protein. Two main groups of protein in gluten are gliadins and glutenins that make nearly 85% of total wheat flour protein (Gennadios 2002).

3.3.2.1.1. Properties of Glutenins, Gliadins and Gluten Complex

Glutenins are polymeric protein fractions which consist of number of subunits linked by intermolecular and intramolecular disulfide bonds (Domenek, et al. 2004). They are linear and alcohol insoluble protein fraction of wheat gluten complex. The amino acid composition of glutenin is shown in Table 3.3. Glutamic acid, proline and other nonpolar amino acids are present at high levels, while basic amino acids like tryptophan are found in small proportions (Krochta, et al. 1994).

Gliadins are single chain polypeptides with low molecular weights including intra molecular disulfide linkages. They are soluble in alcohol and classified into four groups namely α, β, γ, ω, based on electrophoretic mobility under acidic conditions. As can be seen from Table 3.3. that gliadins contain high glutamic acid, proline and have low level of lysine and other basic amino acids. Due to the hydrophobic interactions between the nonpolar amino acid residues and the presence of ionizable basic and acidic groups gliadins posses limited solubility in water. Hydrophobic bonds also lead to globular conformation of gliadin which acts as a plasticizer and contribute to the viscosity to gluten (Krochta, et al. 1994).

Table 3.3. Amino acid composition (moles amino acid per 10^5 g protein) of wheat gliadin, wheat glutenin and wheat gluten (Source: Krochta, et al. 1994)

Amino acid	Wheat Gliadin	Wheat Glutenin	Wheat Gluten
Lysine	5	12-13	9
Histidine	14-15	13	15
Arginine	15	20	20
Aspartic Acid	20	23	22
Threonine	18	26	21
Serine	38	50	40
Glutamic acid	317	278	290
Proline	148	114	137
Glycine	25	78	47

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Table 3.3. (cont.) Amino acid composition (moles amino acid per 10^5 g protein) of wheat gliadin, wheat glutenin and wheat gluten (Source: Krochta, et al. 1994)

Amino acid	Wheat Gliadin	Wheat Glutenin	Wheat Gluten
Alanine	25	34	30
Cystine	5	5	7
Valine	43	41	45
Methionine	12	12	12
Isoleucine	37	28	33
Leucine	62	57	59
Tyrosine	16	23-27	20
Phenylalanine	38	27	32
Tryptophan	5	8	6

Simplified molecular structure of gluten network is illustrated in Figure 3.8. Linear glutenin proteins and globular gliadins play an important role to form gluten complex via covalent and non covalent bonding. Linear proteins interact with each other through disulphide bonds. Smaller globular gliadins fit in the spaces of network and interact with glutenins by noncovalent forces such as Van der Waals interactions (Krochta, et al. 1994).

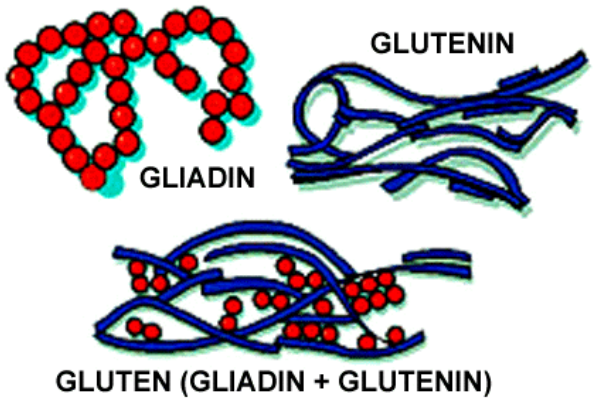


Figure 3.8. Molecular structure of Wheat Gluten
(Source: Landfood 2008)

3.3.2.1.2. Wheat Gluten Films

WG films have been gained much attention due to their barrier properties to O_2 and CO_2 and their rubber-like mechanical properties. WG proteins are insoluble in water and need complex solvent system with heating under acidic and basic conditions for solubility (Gennadios, et al 1997). Adjusting the film solution to acidic or basic conditions contributes to cleavage and reduction of intramolecular and intermolecular disulfide bonds to sulfhydryl groups. By means of using reducing agents such as mercaptoethanol, sodium sulfite and sodium borohydride the reduction of disulfide bonds in gluten solution is promoted. Heating during preparation of film solution, reoxidation and sulfhydryl-disulfide interchange reactions in drying step increase the reformation of disulfide bonds to obtain homogeneous WG films. Adding plasticizer into the film solution is also required since films without plasticizers are very brittle (Domenek, et al. 2004, Krochta, et al. 1994, Gennadios, et al 1997).

Mechanical and barrier properties of WG films have been extensively studied (Palmu, et al. 2000, Micard, et al. 2000). Generally, WG films possess low tensile strength but high elongation at break when compared with other biobased films (Table 3.4.). Water vapor permeability of WG films is comparable with other protein or polysaccharide based films but it is relatively higher than synthetic polymeric films (Table 3.5.). Physical and barrier properties of WG films depend on film formation method, plasticizers, processing conditions and environmental factors like relative humidity and temperature (Gennadios 2002).

Table 3.4. Mechanical properties of WG films and other types of films determined at various temperature (T) and relative humidity (RH) (Source: Gennadios 2002)

Film	Tensile Strength (MPa)	Elongation at break (%)	T(°C)	RH(%)
CA	65.6	30	-	-
LDPE	12.9	500	-	-
MC	56.1	18.5	25	50
FMP	17.1	22.7	25	57
WPI	13.9	30.8	23	50
WG (pH=3)	0.9	260	25	50
CZ	0.4	-	26	50

CA=Cellulose acetate, LDPE= Low-density polyethylene, MC=Methyl cellulose, FMP=Fish myofibrillar proteins, WPI= Whey protein isolate, WG= Wheat Gluten, CZ= Corn zein

Table 3.5. Water vapor permeability (WVP) of WG films and other types of films determined at various temperature (T) and relative humidity (RH) gradient conditions (Source: Gennadios 2002)

Film	WVP(10^{-12} mol/m.s.Pa)	T(°C)	Thickness (mm)	RH Gradient (%)
Starch	142	38	1.19	100/30
SC	24.7	25	-	100/0
CZ	6.45	21	0.2	85/0
HPM	5.96	27	0.019	85/0
WG-Glycerol	5.08	30	0.05	100/0
WG-Oleic acid	4.15	30	0.05	100/0
WG-Carnauba wax	3.9	30	0.05	11/0
WG-Beeswax bilayer	0.023	30	0.09	100/0
LDPE	0.0482	38	0.025	95/0

SC= Sodium caseinate, CZ= Corn zein, HPM= Hydroxypropyl methylcellulose, WG= Wheat Gluten, LDPE= Low-density polyethylene

3.3.2.2. Whey Protein Films

Whey proteins are present in milk and represent nearly 205 total milk proteins. It is a mixture of four main proteins namely β-lactoglobulin (50% wt), α-lactalbumin (20% wt), bovine serum albumin (10% wt) and immunoglobulins (10% wt) (Gallstedt and Hedenqvist 2004). Heat denaturated whey proteins with addition of plasticizers such as PEG and sorbitol provide transparent and flexible films having excellent oxygen, aroma and oil barrier properties at low relative humidity. Although whey protein films have enhanced mechanical properties they show poor barrier to moisture due to their hydrophilic nature (Gennadios 2002).

3.3.3. Lipid Based Films

Films made from lipids have excellent moisture barrier properties because of their tightly packaged crystalline structure (Weber 2000). However, they posses poor self- supporting film formation ability and fairly weak mechanical strength that make them unsuitable for food applications. Acetoglycerides and beeswax are the most common lipids used to produce edible films (Ryu, et al. 2002).

3.4. Application of Biobased Films

Shelf life of foods can be extended by using biobased films from polysaccharides, proteins and lipids. These films act as barrier to gases, vapor, aroma compounds and carriers for antimicrobial and antioxidant agents. Biobased films having good barrier properties could eliminate the moisture loss during storage of fresh and frozen meats. Lipid oxidation and brown coloration in meats can be decreased by biopolymers with low oxygen permeability (Gennadios 2002). Different applications of edible films/coatings in food are displayed in Table 3.6.

Table 3.6. Different applications of edible films/coatings in food
(Source: Haugard, et al. 2001)

Product	Critical Functions of packaging	Value added function	Examples of materials
Meat products			
Fresh meat	moisture barrier	antioxidant effect	
Cured meat	oxygen barrier	antimicrobial effect	
Cooked meat	frying oil barrier	-	alginate, cellulose
Seafood			
Fish	oxygen barrier	antioxidant effect	whey protein
Ready meals			
Pizza	moisture barrier	moisture control	alginate
Fruits and vegetables			
Mushrooms	oxygen barrier	-	alginate
Cabbage	oxygen barrier	reduction of browning	sucrose fatty esters
Strawberries	moisture barrier	retardation of senescence	cellulose based with polyethylene glycol

As an application of active packaging, biopolymers carrying antimicrobial agents are commonly used for reducing the load of spoilage and pathogenic microorganisms at the surface of meat and poultry products (Gennadios, et al. 1997). Many studies have demonstrated that adding antimicrobials into the packaging material is more efficient than directly using on foods because these compounds release themselves from packing material to the surface of foods slowly and in a controlled way thus keeping their high concentration on the product surface for extended period of time (Kristo et al, 2007, Min and Krochta, 2005). Figure 3.9. shows the schematic representation of a preservation method using edible films containing food additives.

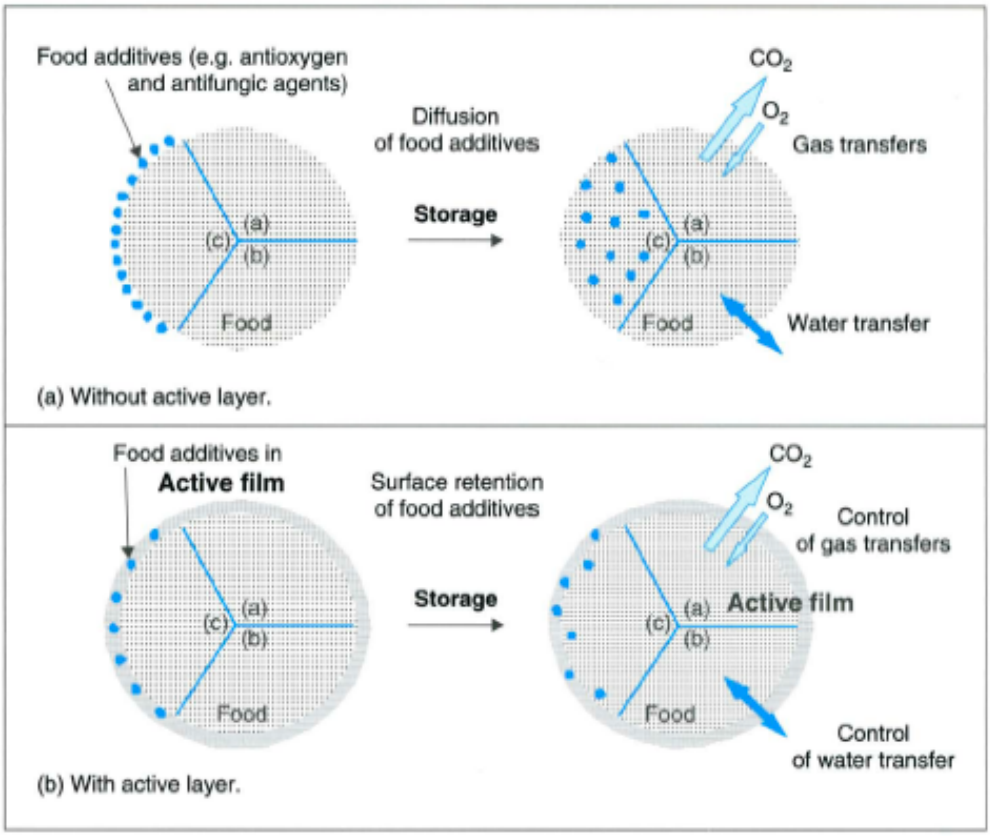


Figure 3.9. Schematic representation of food preservation without (a) or with (b) edible films as active layers (Source: Lacroix and Cooksey, 2005)

Different types of biopolymers and antimicrobial compounds incorporated in edible food packages have been investigated (Table 3.7.). Bacteriocins, especially nisin, are one of the most commonly used antimicrobial agents to prepare antimicrobial films and were added to soy protein, corn zein and alginate films (Padgett, et al. 1998, Padgett et, al. 2000, Cutter, et al. 2001). Min et al. (2005) studied the whey protein isolate film containing lactoferrin, lysozyme and lactoperoxidase system against *S. enterica, E. coli* O157:H7 and *P. commune.* Zivanovic et al. (2005) also examined the antimicrobial properties of chitosan films containing essential oils *in vitro* and on processed meat against *L. monocytogenes* and *E. coli* O157:H7. Ozdemir and Floros (1999) demonstrated the antimicrobial activity of whey protein isolate films containing potassium sorbate against *S. cerevisiae*, *A. niger* and *P. roquefortii*. Inhibitory effects of whey protein films including lysozyme and nisin against *L. monocytogenes*, *S. typhimirium*, *E. coli* O157:H7 were also found (Rodrigues and Han 2000). Elimination of *L. monocytogenes* on ham, turkey breast meat and beef was demonstrated by

incorporating pediocin or nisin into cellulose matrix (Ming, et al. 1997). Antimicrobial biopolymers were generally prepared by incorporating one type of active agent into the film. However, it has been known that some antimicrobial agents might have synergistic effects in inhibiting bacterial growth when they are used in combination. There are several examples of use of combinations of antimicrobials in packaging. Garlic oil, potassium sorbate or nisin was added to chitosan to enhance its antimicrobial activity and it was concluded that antimicrobial activity of chitosan was improved with addition of garlic oil without causing any significant effect on the physical and mechanical properties of the film (Pranoto, et al. 2005). In another study, it was reported that grape seed and green tea extracts enhanced the antimicrobial activity of nisin against *L. monocytogenes* in soy protein coating (Theivendran, et al. 2006).

Table 3.7. Application of biopolymers in antimicrobial food packaging
(Source: Chinnan and Cha 2004)

Biopolymers	Antimicrobial agents	Food
Polysaccharides		
Starch and derivatives	potassium sorbate	strawberry
Cellulose and derivatives		
Cellulose	pediocin	meat
Cellulose based paper	nisin/lacticin 3147	cheese/ham
MC/HPMC-fatty acids	potassium sorbate	water-glycerol
MC-palmitic acid	sorbic acid/ potassium sorbate	water-glycerol
Alginate	glucose oxidase	fish
Alginate	nisin	poultry
Chitosan	acetic/propionic acid	meat
Agar	nisin	poultry
Proteins		
corn zein	Lysozyme/nisin	culture media
	nisin/lauric acid/	culture media
Soy protein isolate	Lysozyme/nisin	culture media
Whey protein isolates	potassium sobate	water-glycerol
Wheat gluten	sorbic acid	water-glycerol

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

Raw materials that were used in preparation of biopolymers were wheat gluten (WG) and methylcellulose (MC). They were obtained from Sigma-Aldrich (Germany). Ethanol (99.5 %) and acetic acid (99.5 %) used as solvents were supplied from Riedel-de Haen (Germany). Glycerol (99.5 %) used as plasticizer to eliminate the film brittleness was purchased from Sigma-Aldrich (Germany). Sodium sulfite (98%) was also supplied from Sigma-Aldrich (Germany). Magnesium nitrate used to control relative humidity was supplied by Tekkim (Turkey). Antimicrobial agents namely, activated lactoferrin (ALF) donated by DMV International, USA and Pimalac® was used as a natamycin (NA) source. For the application of films, Kashar cheese was purchased from local supermarkets (Izmir, Turkey).

4.2. Microbial Cultures and Growth Conditions of Microorganisms

Escherichia coli O157:H7 (NCTC12900), *Listeria monocytogenes* (NCTC 11994) and *Salmonella enteritidis* (NCTC12694) were obtained from National Culture Type of Collection (United Kingdom). *Aspergillus niger* was isolated from onion skin in Plant Protection Department of Mustafa Kemal University (Hatay, Turkey) and *Penicillium roquefortii* DBCI-1 was isolated from Danish blue cheese in Food Engineering Department of Izmir Institute of Technology (Izmir, Turkey). Identities of both fungi were confirmed with microscopy and morphological analysis. Media used for growth of microorganisms and microbial analysis, nutrient agar (NA), brain heart infusion (BHI) (broth and agar) and tryptic soy broth (TSB), were purchased from Fluka (Germany) and potato dextrose agar (PDA) and dichloran rose bengal chloramphenicol agar (DRBC) were obtained from Merck (Darmstadt, Germany). Lauria broth (LB) was prepared by mixing 5 g yeast extract (Acumedia), 10 g tryptone (Fluka) and 5 g NaCl (Riedel-deHaen) per liter.

Three food borne pathogenic bacteria and two spoilage molds, which are typically meat and cheese product contaminants were used in this study. *E. coli* O157:H7*, L. monocytogenes and S. enteritidis* were grown LB, BHI and TSB, respectively at 37^{o}C without shaking and agar plates were kept refrigerated until used. *A. niger and P. roquefortii* were grown on PDA slants at 30^{o}C. Subculture was carried out weekly to maintain microbial viability and all the strains were stored at -80^{o}C in their media containing 20 % glycerol throughout the study.

4.3. Preparation of Bacterial Cultures

To prepare the microbial suspension for antimicrobial test of biopolymers and paper pieces including antimicrobial agents, a single of bacteria from agar plates was taken and inoculated into sterile 10 ml tubes containing 4 ml proper media for each bacterial strain. Then, media containing bacteria was incubated at 37^{o}C for nearly 16 hours. After adjusting the optical density (OD) of culture to desired value at 600 nm via sterile media, 2% bacterial culture was transferred to fresh media. Cultures were incubated again for 5.5 hours, 3 hours and 2.5 hours for *L. monocytogenes, S. enteritidis* and *E.coli* O157:H7, respectively to get the bacteria in mid-log phase according to their growth curve. Serial dilution was made by proper media to produce desired inoculum level. To confirm the results, all bacteria were enumerated on agar plates after 24 hours of incubation at 37^{o}C.

4.4. Preparation of Fungal Cultures

Fungal spores were collected by sterile 0.1% peptone water from 3-5 day old subcultures of *A. niger* and 5-7 day old cultures for *P. roquefortii*. Homogenized solution was transferred to sterile tube and in order to liberate the spore, test tube was vigorously shaken nearly 5 min. Spore load was determined by means of counting on Thoma slide. The culture suspension of mold was adjusted to $1x10^{4}$ and $1x10^{6}$ spore/ml for *A.niger* and *P. roquefortii*, respectively with 0.1% peptone water before in use.

4.5. Preparation of Rosemary Extract

Rosemary samples *(Rosemarinus officinalis*) were collected from their natural habitat and rinsed with sterile deionized water. After separating leaves from their stem, extraction procedure with some modification from Madsen et al. (1998) was immediately applied. 12 g leaves were homogenized in 70 ml absolute ethanol at 26000 rpm (Heidolph Silent Crusher M Homogenizer, Germany) for 5 min. Obtained solution was stirred for 30 min in dark and centrifugated at 5000 rpm (Nuve NF 615, Turkey) for 5 min. Supernatant was collected and 30 ml ethanol was added to precipitate and same procedure was repeated twice. 20 ml ethanol was used in the last extraction step. Collected supernatant was evaporated under vacuum at 40^{o}C for approximately 1 hour with a rotary evaporator (Heidolph Laborato 4000, Germany). Obtained rosemary extract (RE) was vacuum filtered through 5 μm pore sized cellulose nitrate filter (Sartorius) Finally, extract was serially diluted to defined concentrations by sterile deionized water.

4.6. Testing the Effectiveness of Antimicrobial Agents by Agar Disc Diffusion Method

The agar disc diffusion method was applied for the determination of antimicrobial activities of ALF, RE and NA against tested bacteria and molds. Whatman no: 1 paper discs were cut into 1cm x 1cm squares and sterilized prior to use. 20 μl antimicrobial solutions were pipetted into 1 cm^2 square pieces and then pieces were placed on agar plates which had been previously seeded with 0.1ml of inoculum containing indicator microorganisms. After the incubation period, antibacterial and antifungal activity of compounds was determined by measuring the zones formed around discs. Diameter of pieces was also included in inhibitory zone diameter.

4.6.1. Agar Diffusion Assay for Bacteria

According to recipe proposed by manufacturer, 4% ALF was prepared by dissolving 0.54 g ALF in 10 ml sterilized deionized water. 3, 2, 1.5 and 1 % ALF concentrations were prepared by serial dilution with sterile deionized water. After

preparation of RE; 60, 40, 30, 20 and 10 % RE concentrations were obtained by serial dilution with sterile deionized water. NA was dissolved in glycerol/ethanol (1:1, v/v) solution and appropriate concentration with a range of 250-5000 ppm were utilized during the experiment.

Combinations of 50% RE+2% ALF, 25% RE+3% ALF, 35% RE+1% ALF and NA+RE combination (25-2500 ppm NA+50% RE) were also prepared to evaluate the synergistic activity of the compounds. Controls were prepared by absorbing deionized water, peptone water, glycerol, ethanol and glycerol + ethanol to paper pieces.

After preparation of antimicrobial solution, 100 µl bacterial cultures containing $1x10^6$ cfu/ml were spread on agar plates and paper discs including controls or active agents were placed at the centre of inoculated plates under a biological safety cabinet as explained before. Then, they were incubated at 37^{o}C for 24h and the diameters of the inhibition zones were measured with a caliper.

4.6.2. Agar Diffusion Assay for Molds

Same concentration and agar diffusion technique explained above were also used to determine the antifungal properties of ALF, RE, NA and their combinations against *A. niger* and *P. roquefortii.* Pieces were placed on PDA agar plates seeded with either 10^4 spores/ml *A. niger* or 10^6 spores/ml *P. roquefortii*. *A. niger* containing plates were incubated for 3 days at 30^{o}C while incubation time for plates with *P. roquefortii* was 6 days at 30^{o}C. The diameter of inhibitory zone surrounding pieces was then measured.

4.7. Preparation of Biopolymers

4.7.1. Wheat Gluten Films

Wheat gluten films were prepared according to a method by Bohatier-Pochat, et al. (2006) with some modifications. 15 g of wheat gluten was dissolved in 31.5 ml absolute ethanol with mixing. Then, 0.03 g sodium sulfite, 3 g glycerol and 63 ml deionized water were added to the solution and homogenized with a magnetic stirrer for nearly 10 min pH of film solution was set to 4 with acetic acid and solution was mixed

and heated to 70°C on magnetic stirrer. 10 g of film solution (fs) was spread onto 8.5 cm diameter polystyrene petri dishes and dried at 30°C.

4.7.2. Methylcellulose Film

A procedure by Turhan and Sahbaz (2004) with some modifications was used for the preparation of methyl cellulose (MC) films. 3 g MC was mixed with 50 ml deionized water. 50 ml ethanol was added and homogenized. After addition of 1 ml glycerol, solution was heated to 80°C. 10 g of fs was spread onto polystyrene petri dishes and dried at at 30°C for nearly 2 days.

4.8. Incorporation of Antimicrobial Agents into Films

The three antimicrobial agents, ALF, NA and RE were incorporated into film solutions (fs) at various concentrations to test antimicrobial efficacy and physical nature of the films. To eliminate the adverse effect of the temperature all antimicrobials were added to film solutions after cooling the solutions to a temperature between 50 – 55°C. ALF with the concentrations of 0.03-0.05-0.07 g ALF /10 g fs was obtained by adding it as powder form to film solution. For the preparation of natamycin (NA) containing films, NA in powder form was added to films just before spreading film solution onto petri dishes. Concentrations of NA were in the range of 0.2-40 mg/10 g fs. RE containing films were obtained by replacing a certain volume of ethanol and water in film formulation with RE. To prepare 10 g film solution containing 3 ml RE, 2.3 ml water and 0.7 ml alcohol were reduced from original film solution recipe and 3 ml RE were added to film solution before spreading onto petri dishes. NA+RE and ALF+RE containing films were made according to procedures explained above. Dried films were peeled off and stored in desiccators at 50% RH and 25°C until further evaluation.

4.9. Determination of Antimicrobial Properties of Biopolymers Containing Antimicrobial Agents

Antimicrobial activity test of biofilms was carried out using agar diffusion method. Films were cut into 1cm x 1cm squares using a scissor. Then, they were placed

on agar plates previously inoculated with microorganisms. Incubation period and microbial load for both bacteria and mold were same as the paper disc diffusion method which was explained before. WG or MC films which are not containing any active agent were used as the controls. All tests were performed at least three times.

4.10. Film Thickness

An electronic digital micrometer (Shan Electronic, China) was used to measure the film thickness to nearest 0.001 mm. Thickness of each film (mm) was measured at room temperature and expressed as an average of 10 random measurements. Mean values were used to determine the water vapor permeability and mechanical properties of tested films.

4.11. Determination of Mechanical Properties of Films

Mechanical properties of the films were tested according to ASTM Method D882 by using Mechanical Testing Machine (AG-I 250 kN, Schimadzu, Japan). Films were cut into 25 mm x 100 mm strips and preconditioned at 50% RH in a desiccator for 48 hours before the measurement of mechanical properties. Initial grip separation and cross-head speed were set to 50 mm and 50 mm/min, respectively. Force and elongation values were recorded and the values of tensile strength (TS), percentage elongation at break (EB) and elastic modulus (EM) were calculated from the stress-strain curve (Figure 4.1.). TS was calculated by dividing the maximum load for breaking the film by cross-sectional area (thickness x width). EB was calculated by dividing film elongation at the moment of rupture by the initial gauge length of samples multiplied by 100%. EM was calculated from the slope of the initial linear region of the stress-strain curve. At least 6 specimens for each film type were tested.

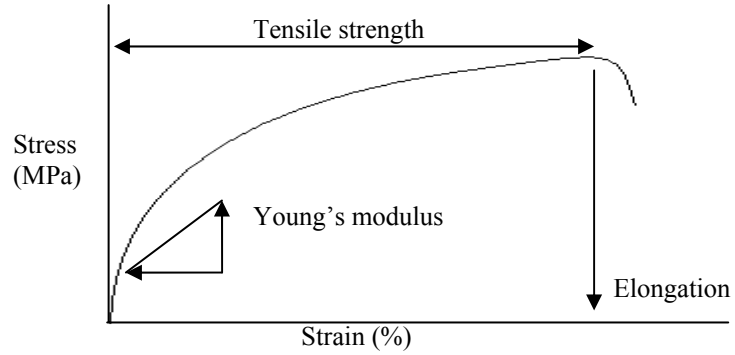


Figure 4.1. Typical (stress)-force (strain)–deformation curve for calculation of mechanical properties of films

4.12. Measurement of Water Vapor Barrier Properties of Films

Water vapor permeability (WVP) of films was measured according to WVP Correction Method (McHugh, et al. 1993). Film discs, previously conditioned at 50% relative humidity in desiccators at least 48 hours before testing, were sealed to glass jars containing deionize water and the cups were placed in air–circulated desiccators including saturated solutions of $MgNO_3$ (50% RH). Set-up used in measuring WVP is shown in Figure 4.2. Desiccators were kept in a room at 25^oC during the experiment. The area of jar mouth was 17.34 cm^2, and the jar depth was 7 cm. The air gap between the film and water in the jar was 1.4 cm. Fans operating at speeds of 2 m/sec (app. 120 m/min) were also placed in the desiccators and fan speed was measured with an anemometer (Turbo Meter, USA). Steady state was achieved within 2h and each cup was weighed at least 5 times at 2h intervals. Weight changes of glass jars were measured and plotted against time. Three replicates of each film were tested and WVP Correction Method was used in calculation of relative humidities at the films undersides and water vapor permeability values.

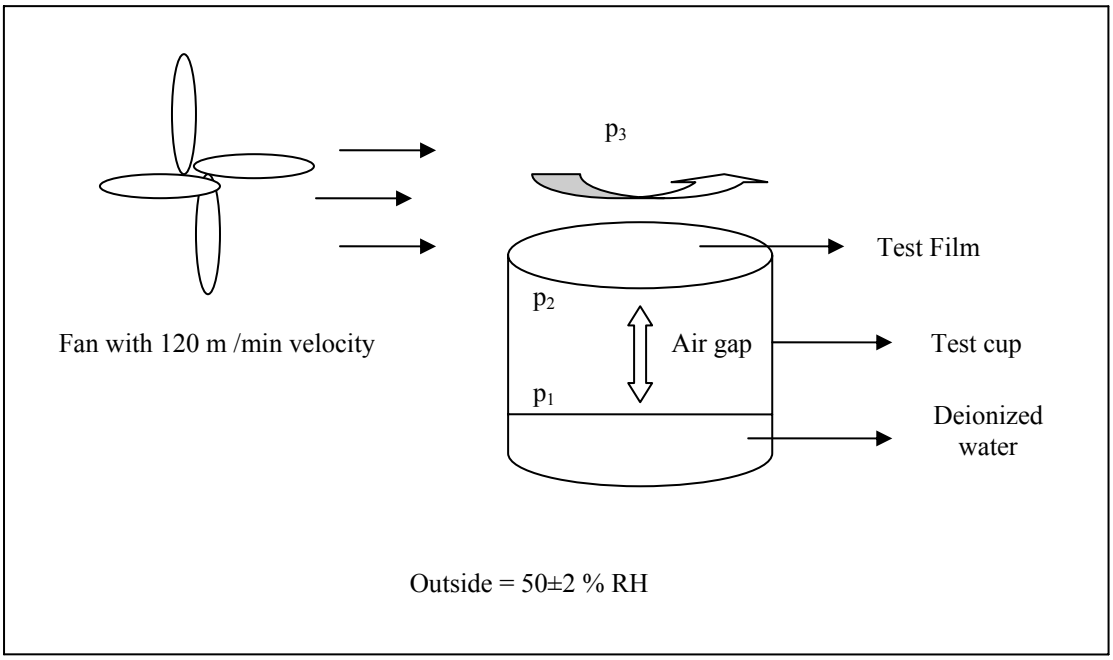


Figure 4.2. Schematic representation of modified test cup method

Water vapor transmission rate (WVTR) was calculated using Equation (1) (ASTM E96, 1980). Regression analysis of weight loss as a function of time was performed and correlation coefficients for all calculated data were ≥ 0.99.

$$WVTR = \frac{Slope}{Film\,area} \tag{1}$$

where Slope is obtained from Weight loss vs. Time graph

In water vapor permeability (WVP) correction method, Equation (2) provides the calculation of the corrected water vapor partial pressure at the film inner surface (p_2)

$$WVTR = \frac{P * D * Ln[P - p_2)/(P - p_1)]}{R * T * Z} \tag{2}$$

where P= Total pressure; D= Diffusivity of water through air at 25 ^{o}C; R=Gas law constant (82.057*10^{-3} m^3-atm/kgmol-K; T=Absolute temperature (298K); Z=Air gap; p_1=Water vapor partial pressure at solution surface; $p_{2=}$ Corrected water vapor partial pressure at film inner surface in cup.

After calculation of p_2, water vapor permeance can be obtained using Equation (3)

$$Permeance = \frac{WVTR}{p_2 - p_3} \quad (3)$$

where p_3= Water vapor partial pressure at film outer surface in the cabinet.

Finally, WVP of the film could be calculated from Equation (4)

$$Permeability = Permeance * Thickness \quad (4)$$

Eq (2), (3) and (4) is defined as the WVP Correction Method.

4.13. Fourier-Transform Infrared Spectroscopy (FTIR) Measurements

Infrared spectra of films were obtained in 4000-650 cm^{-1} range with a Perkin Elmer Spectrum 100 FT-IR spectrometer (Perkin Elmer Inc., Wellesley, MA) equipped with a deuterated tri-glycine sulphate (DTGS) detector. A horizontal attenuated total reflectance (HATR) sampling accessory (ZnSe crystal) was used to collect the spectral data of films. The resolution was set at 4 cm^{-1} and the number of scans collected for each spectrum was 64.

4.14. Scanning Electron Microscopy (SEM) Analysis

Films were conditioned at 50% relative humidity in a desiccator at least 48 hours before SEM analysis. Films were coated with 100 - 200 Å thickness of gold. Cross section of films were scanned with Phillips XL-30S FEG electron microscope.

4.15. Application of Biopolymers Containing Antimicrobial Agent in Food

Antifungal activities of NA added biopolymers against *A.niger* and *P.roquefortii* inoculated onto the surface of Kashar cheese were also investigated.

4.15.1. Sample Preparation and Inoculum

Cheese samples were purchased from a local supermarket and transported immediately to laboratory in an insulated box containing ice packs. Cheeses with 1 cm thickness were cut into 2 cm circular diameter (approximately 20g) using a sterile

beaker.Then each surface was treated under UV light (254nm) at a distance of 15 cm for 10 minutes. Sliced cheeses were dipped into either 10^5 spores/ml *A. niger* and *P.roquefortii* for 2 min and allowed to drip excess inoculation. Then, each surface was dried for 10 min under laminar hood. Dried cheeses were placed between nearly 4.5 cm circular diameter film pieces. Assembly was placed into sterile petri dishes, sealed with zipper plastic bags and stored at 10^{o}C in a refrigerator for 30 days. Samples used during the experiment were as follows: 1) no treatment 2) UV treated 3) UV treated and inoculated 4) UV treated + inoculated + control films 5) no treatment + control films 6) UV treated + inoculated + natamycin containing films (2-5-10-20 mg/10g film solution).

4.15.2. Microbiological Analysis

Cheese samples were taken after 2 hours on day 0 and again after 10, 20, 30 days of storage at 10 ^{o}C for microbiological analysis. Films were separated from cheese slice with sterile forceps and cheese samples were mixed with approximately 180 ml 0.1% peptone water in a sterile stomacher bag and homogenized using a Stomacher (BagMixer, France) at 230 rpm for 2min. Appropriate dilutions with 0.1% peptone water were made and 0.1 ml sample was spread on DRBC agar plates and incubated at 30 ^{o}C for 2 d. All tests were carried out in duplicate.

4.16. Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using the MINITAB (version 14.10). Means were compared using Fisher Least Significant Difference (LSD) method at p=0.05.

CHAPTER 5

RESULTS AND DISCUSSION

In the first part of this study, antimicrobial properties of ALF, RE and NA were assessed against several microorganisms by agar disc diffusion method. Moreover, antimicrobial MC and WG films were prepared by adding these agents into film solution and the inhibitory activity of films was measured based on clear zone formed around square film pieces placed on agar medium. In the second part of the study, films having antimicrobial activities were examined in terms of their mechanical (TS, EB and EM) and barrier properties (WVP) by using ASTM D882 and WVP correction method, respectively. Also, topology of the antimicrobial films and possible interactions between antimicrobial agents and film structure were investigated by scanning electron microscopy and Fourier-transform infrared spectroscopy. Finally, antimycotic activity of MC and WG films containing NA were studied on Kashar cheese during 30 days storage at 10^{o}C.

5.1. Antibacterial Properties of ALF, RE and NA by Agar Disc Diffusion Method

Antibacterial activities of ALF (4, 3, 2, 1.5, 1% (wt/vol)), RE (60, 40, 30, 20, 10% (vol/vol)), NA (250-5000 ppm), ALF-RE combinations (50% RE+2% ALF, 25% RE+3% ALF, 35% RE+1% ALF) and NA-RE combination (25-2500 ppm NA+50% RE) were tested against *L. monocytogenes*, *S. enteretidis* and *E. coli* O157:H7 by agar disc diffusion assay. It was found that none of these agents and their combinations showed any inhibitory effect against tested bacteria during 24 h incubation at 37 ^{o}C. In a previous study, Eroglu et al. (2007) investigated the antimicrobial activities of ALF and RE against food borne pathogenic bacteria by microtiter plate assay. MIC of ALF was found as 0.1%, 0.5% and 1% against *L. monocytogenes*, *S. enteretidis* and *E. coli* O157:H7, respectively. RE also had inhibitory effect against *L. monocytogenes* at the concentration of 15% (v/v). Synergistic activity between RE (10-20%) and ALF (0.3-

0.5%) was determined against *E. coli* O157:H7. Naidu (2002) also determined the antibacterial activity of ALF at the concentration of 1% against *E. coli* O157:H7 by optical density measurement. In the study of Del Campo et al. (2005) commercial RE dissolved in ethanol had no effect on the gram negative bacteria such as *S. enteritidis* while growth of the gram-positive bacteria, *L. monocytogenes*, was inhibited at the concentration of 0.5% in TSB broth. Antilisterial activity of *R. officinallis* (≥ 5 %w/v) also revealed by Pandit and Shelef (1994). Although NA is effective on molds and yeasts there is no conclusive report on the possible action of NA on bacteria. Therefore our results are consistent with other researchers (Stark 2003, Mohamed, et al. 2005).

In summary, antimicrobial properties of ALF and RE had variations from one study to another in literature and this might be due to the differences in antimicrobial activity testing methods. Results of antimicrobial activity tested in solution and diffusion through a solid disc might differ. Agar disc diffusion test was used in this study and this test is commonly used for examining the antimicrobial activity regarding the diffusion of the agents through water-containing media. It is well known that diffusion depends on the size, shape and polarity of the diffusing molecule (Pranoto, et al. 2005). ALF is a protein which is immobilized on galactose rich polysaccharide or carrageenan, and high molecular weight of ALF could restrict its diffusion into the media (Naidu 2002).

As far as RE was concerned, various procedures were used for the extraction of rosemary in the previous studies in literature and antimicrobial activity showed variations depending on the extraction method and solvent. Çeliktas et al. (2007) reported that methanol extracts of RE exhibited very low antimicrobial activity against several bacteria including *E. coli, Staphylococcus aureus*, *Proteus vulgaris*, and *Bacillus subtilis* by disc diffusion method. According to another study, while supercritical fluid extract of rosemary had a strong antioxidant activity, its antimicrobial activity was low (Leal, et al. 2003).

5.2. Antifungal Properties of NA, RE and ALF by Agar Disc Diffusion Assay

Same active agents used in disc diffusion method for bacteria were also tested against *A. niger* and *P. roquefortii* which are the prevalent spoilage organisms in dairy

products. Table 5.1. shows the effect of NA concentration on growth inhibition of *A. niger* and *P. roquefortii.*

Table 5.1. Agar disc diffusion results for different NA concentrations when applied on paper disc form on *A. niger* and *P. roquefortii*

NA conc. (ppm (mg/cm^2))	Diameter of inhibition zone (cm)	
	A. niger	*P. roquefortii*
0 (0)	+	+
250 (0.005)	+	+
500 (0.01)	+	+
750 (0.015)	1.5±0.0 [a]	3.02±0.11[a]
1000 (0.02)	1.66±0.12 [a]	3.18±0.24[a]
1250 (0.025)	2.62±0.27 [b]	4.17±0.72[b]
2500 (0.05)	3.49±0.32 [c]	4.30±0.24[b]
5000 (0.1)	3.98±0.3 [d]	5.32±0.43[c]

+ no inhibition, [a-d] same letters show that there is no statistical difference between different NA levels at P>0.05

NA containing paper discs demonstrated inhibition zones ranging from 1.5 to 3.98 cm at the concentration range of 750 ppm-5000 ppm against *A. niger*, and 3.02 to 5.32 cm for *P. roquefortii.* MIC values of NA for *A. niger* and *P. roquefortii* are 750 ppm. These values are higher than those reported by Stark (2003). On contrary to agar disc diffusion assay used in our study, antifungal activity was tested directly spreading a certain volume of NA suspension on agar in the previously mentioned study. In addition, commercial NA that was used in this study contains 50% NA and 50% lactose. Stark (2003) also reported that 0.01-0.02 mg/cm^2 NA is effective against the molds during surface application. In terms of surface area, MIC value of NA against *A. niger* and *P. roquefortii* is found as 0.015 mg/cm^2 in our study. This value was in the range of that was reported by Stark (2003). MIC value of NA for *P. roquefortii* was the same for *A. niger* although NA was tested against a higher spore load in the case of *P. roquefortii.* With increasing NA concentration, diameter of inhibition zone also increased (Figure 5.1. and 5.2.).

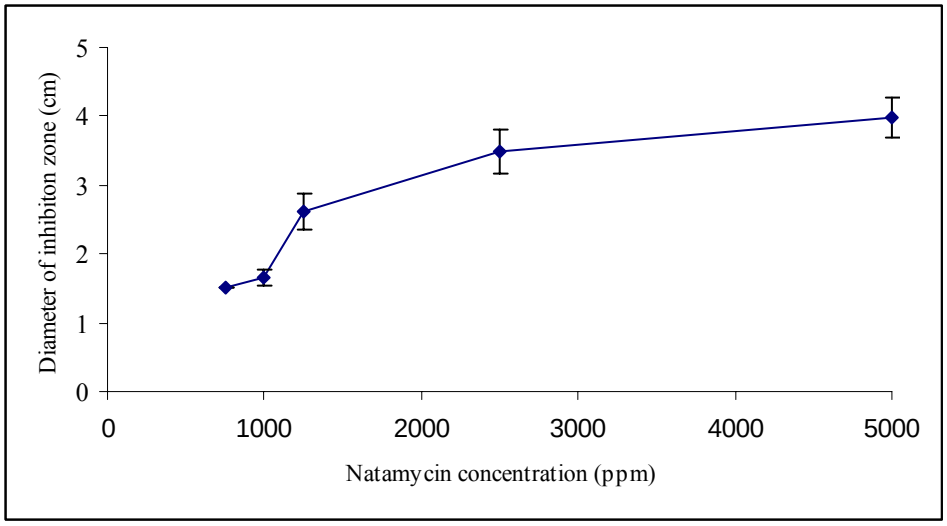


Figure 5.1. Effects of paper discs containing 750-5000 ppm natamycin on the growth of *A.niger* (10^4spore/ml)

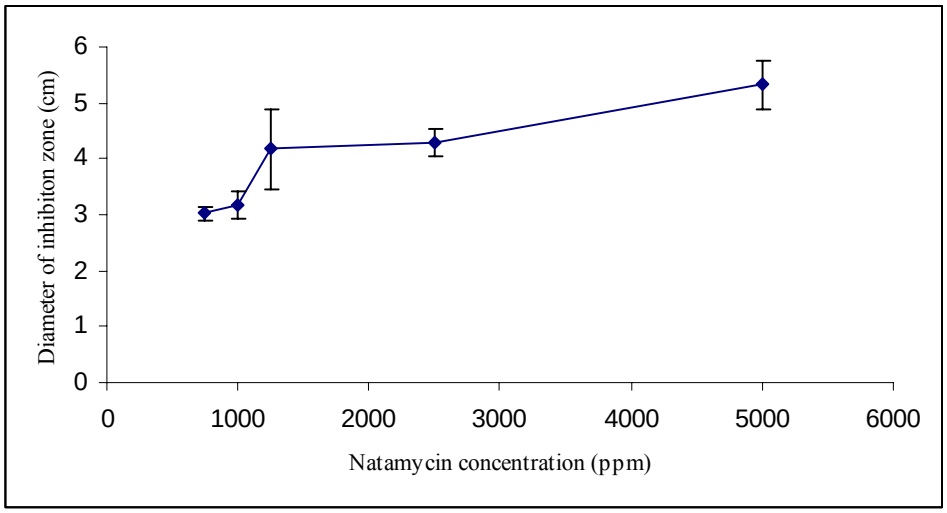


Figure 5.2. Effects of paper discs containing 750-5000 ppm natamycin on the growth of *P.roquefortii* (10^4spore/ml)

Typical inhibition of *A. niger* and *P.roquefortii* growth by paper disc containing NA is shown in Figures 5.3. and 5.4.

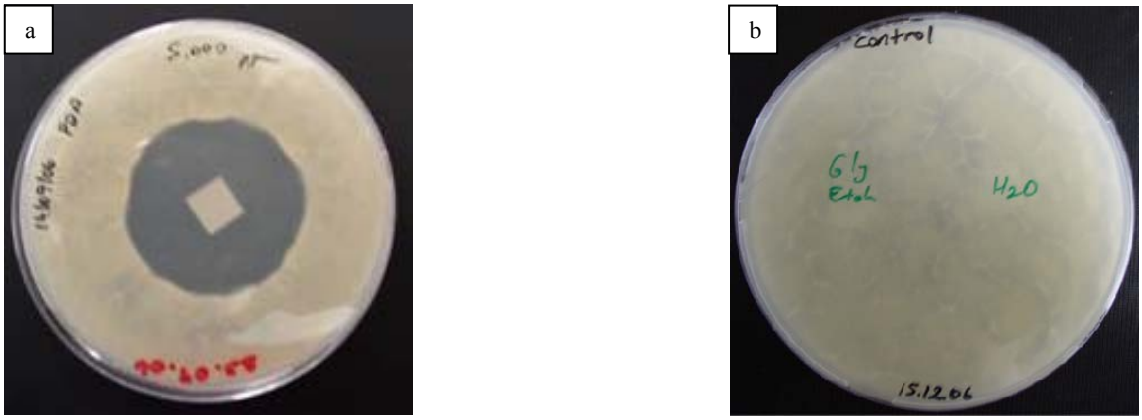


Figure 5.3. Inhibition of *A.niger* by paper disc a) 5000ppm NA b) Control discs

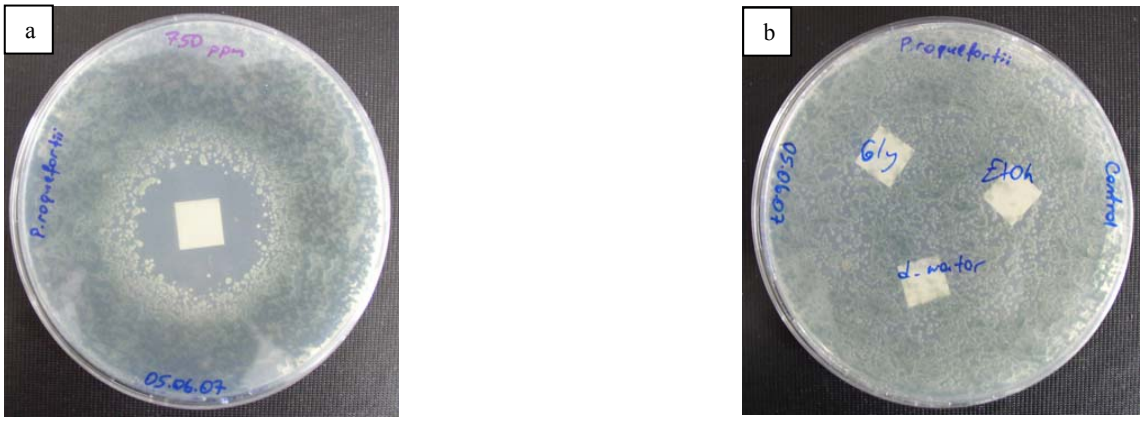


Figure 5.4. Inhibition *of P.roquefortii* by paper discs a) 750 ppm b) Contol discs

While NA was very effective against both fungi, RE and ALF did not show any inhibitory action against *A. niger* and *P. roquefortii*. Effect of several plant extracts and their constituents against molds and yeasts also have been studied in several studies in literature (Viuda-Martos, et al. 2007, Stark 2003). On contrary to our results, it was reported that commercial rosemary extract dissolved in ethanol slowed the growth of *P. roquefortii*, and antibacterial activity was strongly influenced by media composition such as pH and salt content (Del Campo, et al. 2000). Antifungal effectiveness of lactoferrin digest against spore germination and mycelial growth of *Penicillum* sp. isolated from bottled water was determined by 96 well microplate (Gesualdo, et al. 2001). Oregano powder (1-2%w/v) and its essential oil (0.05, 0.025) also showed inhibitory effect on *A. niger* and *P. roquefortii* (Akgul and Kivanç 1988). Abdel-hafez and El-Said (1997) reported the effectiveness of garlic extract up to 0.25% (v/v) to

inhibit the growth of *A. flavus, A. fumigatus, A .niger P. chrysogenum* and *P. citrinum,*. Differences between the results of this study and studies reporting inhibitory action might be result from extraction procedure.

5.3. Antibacterial Properties of MC and WG Films Containing ALF RE and NA

To prepare the antimicrobial films, temperature of both film solutions is decreased to between 50 and 55^{o}C to avoid destruction of the tested agents. ALF and several plant based extract are susceptible to temperature abuse (Naidu 2002). Marques, et al. (2008) revealed that garlic and oregano exhibited strong antimicrobial activities at low temperature against *S. enterica.* It is also well known that exposures in excess of one hour at temperature above 120^{o}C impair the antimicrobial properties of NA (Lück and Jager 1997, Raab 1972).

Antibacterial activities of MC and WG films containing different concentrations of ALF, RE and NA were tested against *L. monocytogenes*, *S. enteretidis* and *E. coli* O157:H7. Effect of ALF with concentrations of 0.03, 0.05 and 0.07g ALF/10g fs was investigated against tested microorganisms by agar diffusion method. After 24h incubation, it was not found any inhibitory effect of WG and MC films against tested bacteria. As explained before, the lack of effectiveness is possibly related to limited diffusion of the agent. Antimicrobial properties of edible films are affected by the molecular size and shape of added agents into the film network. For the incorporation of the compounds into a polymer the molecular weight of antimicrobial agents also need to be taken into consideration since it affects the rate of diffusion in the polymer (Cooksey 2000). Min and Krochta (2005) studied the antimicrobial properties of whey protein films incorporated with lactoferrin (LF) and lactoferrin hydrolysate (LFH) having 78000 Da and 10000-25000 Da molecular weight , respectively. It was stated that these agents diffuse more slowly than other natural antimicrobial agents such as nisin and pediocin which posess molecular weights ranging from 3000 to 5000 Da, thus, limiting their effectiveness on the target microorganism. Furthermore, possible interaction between added agents and film network affects the antimicrobial action of films. Pranoto et al. (2005) also found that chitosan films incorporated with potassium sorbate (PS) did not show inhibitory zone against *E. coli* and *S. typhimurium* because of

chemical interactions between PS and chitosan films. Type of plasticizers and their concentration also have an effect on diffusion of active agents from polymer to the food matrix. Adding more plasticizer results in rise in diffusion rate due to increase of free volume of polymer (Min and Krochta 2005, Sothornvit and Krochta 2000). MC films with and without ALF did not show inhibitory effect against tested bacteria. WG films containing ALF and control films produced inhibition zone against *E. coli* O157:H7 at the end of 11 h incubation at 37°C but antimicrobial activities of tested films disappeared at the end of 24 h incubation (Figure 5.5. and 5.6.). *L. monocytogenes* and *S. enteritidis* were also not affected from WG films.

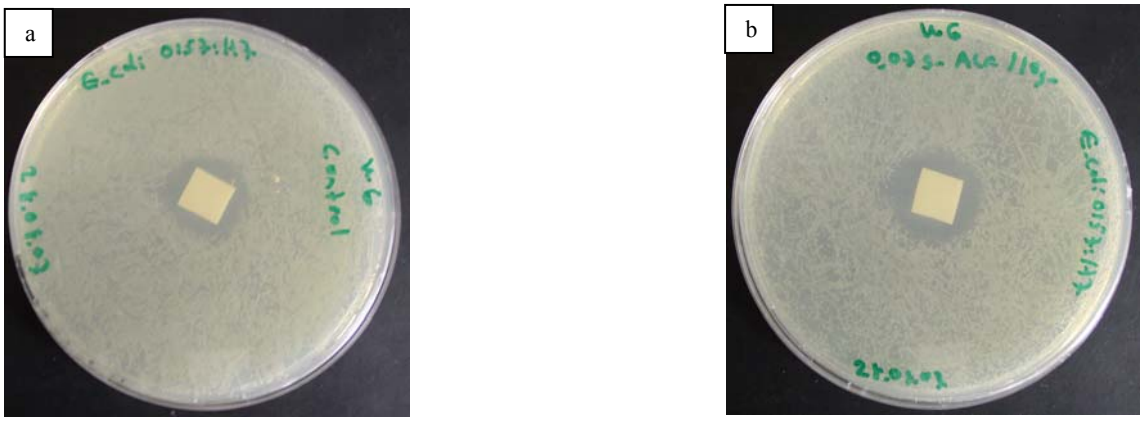


Figure 5.5. Inhibitory effect of WG films against *E.coli* O157:H7 at the end of 11 h incubation at 37°C a) WG control films b) WG film containing 0.07gALF/10g fs

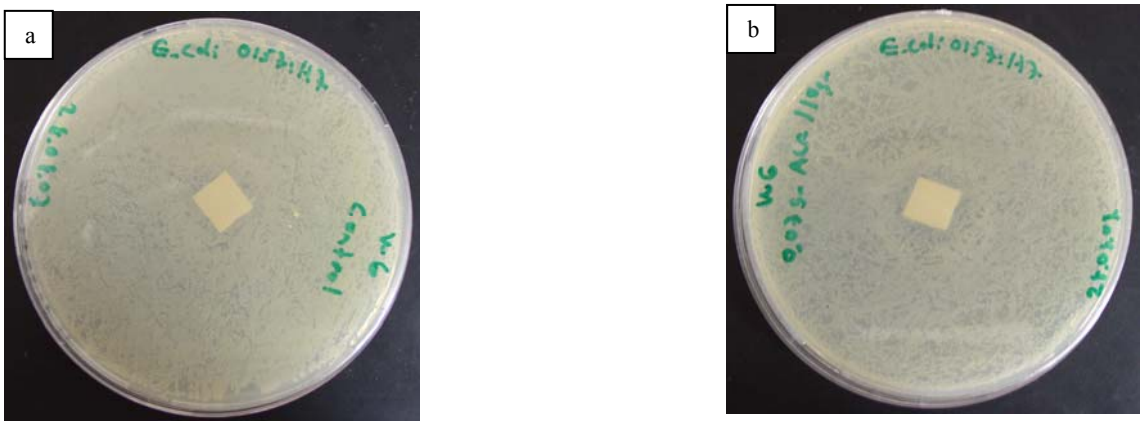


Figure 5.6. Inhibitory effect of WG films against *E.coli* O157:H7 at the end of 24h incubation at 37°C a) WG control films b) WG film containing 0.07 g ALF/10g fs

Inhibitory effect of WG control films against *E. coli* O157:H7 is attributed to presence of small amount of residual acetic acid used for film preparation (Zivanovic, et al. 2005). It is well known from the literature that *L. monocytogenes* has ability to survive under adverse conditions and *Salmonella* sp is more resistant than *E. coli* strains in terms of pH changes in agar media (Min, et al. 2005). In addition, high amount of ALF (0.05g ALF/10g fs) in both film solutions resulted in cracks, dents and production of non-homogeneous films with small particles which can be observed visually in film structure (Figure 5.7.).

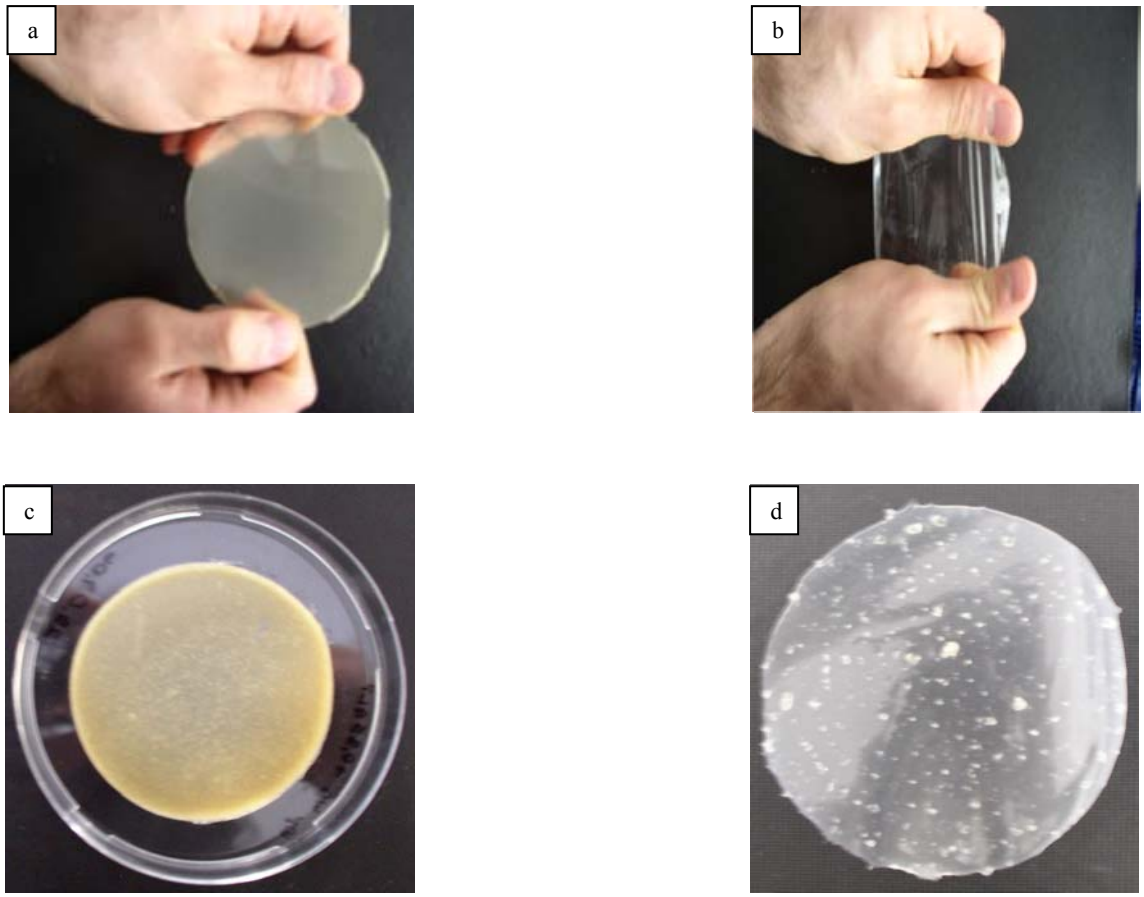


Figure 5.7. a) WG control film b) MC control film c) WG film containing 0.05 g ALF/10g fs d) MC film containing 0.05 g ALF/10g fs

This might be attributed to interaction of ALF with polymer structure and molecular structure of ALF. Incorporation of nisin into the whey protein isolate films caused the rearrangement of disulfide and hydrophobic bonds or more protein-protein interactions leading to changing of mechanical properties of film (Ko, et al. 2001).

Higher amounts of nisin also caused the WG films to be brittle due to the increase in intermolecular interaction between adjacent polymer chains in film (Ko, et al., 2001). RE and NA incorporated MC and WG films (1.5 ml and 3 ml RE /10g fs) at all tested concentration did not produce inhibitory effect against bacteria used in this study. Seydim and Sarikus (2006) prepared whey protein isolate films with essential oil of rosemary and tested its antimicrobial activity against *E. coli* O157:H7, *S. aureus, S. enteritidis, L. monocytogenes* and *L. plantarum* on agar medium. It was found that films with rosemary did not demonstrate antimicrobial activity against tested microorganisms.

5.4. Antifungal Properties of MC and WG Films Containing ALF, RE and NA

Antifungal activities of WG and MC films containing ALF, NA and RE against *A. niger* and *P. roquefortii* were also investigated. WG and MC films containing ALF was not found effective against molds. NA, RE and their combinations were also incorporated into WG and MC films, and their antifungal properties were tested. Table 5.2. and 5.3. list the diameter of fungal inhibition zone for WG and MC films containing various concentrations of NA and NA+RE combinations. Examples of inhibition zones formed around biopolymers are also shown in Figure 5.8. and 5.9. NA added films were very effective in inhibiting the growth of both molds. MIC values of NA for both WG and MC films were 2 mg/10g fs against *A. niger* and 1 mg/10g fs against *P. roquefortii*. Difference between MIC values of both films against tested microorganisms could be explained by cell wall properties of molds. Chitin makes up to 45% of the cell wall of *A. niger* and *Mucor rouxii* while the cell wall of *P. notatum* contains 20% chitin. Therefore**,** NA easily disrupts thin cell wall of *P. roquefortii* while higher concentration of NA was effective on *A. niger* which has thick cell wall in terms of amount of chitin (Roller and Covill 1999). As NA concentration increased from 2 to 40 mg/10g fs the inhibition zone diameter also increased from 2.84 to 3.99 cm for MC film and 2.76 to 4 cm for WG films on agar medium for *A. niger*. Inhibition zone diameters of *P. roquefortii* varied between 2.34-5.06 cm and 2.59-5.30 cm for WG and MC films, respectively (Table 5.2. and 5.3.). NA inhibition zone diameters were very close and NA MIC values were the same for WG and MC films. Although MC is a carbohydrate and WG is a protein based film, chemical structure of the film did not affect the release

behavior of NA from the films. There was a leveling out at inhibition zone diameters around 10 mg NA/10g fs meaning that adding more than this level of NA is unnecessary. Pranoto et al. (2005) also reported that increasing the level of nisin and garlic oil at higher concentrations did not contribute to the inhibitory effect of chitosan film owing to the maximum capability of chitosan polymer. Our results indicated that MC and WG films containing NA are more effective against slow growing mold (*P. roquefortii*) than fast growing mold (*A. niger*). Weng and Chen (1997) also determined that sorbic anhydride incorporated polyethylene films were effective in inhibiting *A. niger* and *Penicillium sp.* Benzoyl chloride modified ionomer films displayed inhibition zones on PDA that had been inoculated with *Aspergillus* sp and *Penicillium* sp. (Matche, et al. 2006). Coma et al. (2005) showed the inhibitory activity of chitosan based edible coatings and films against *A. niger*.

When it is desired to compare MIC values for paper discs and films, it is more convenient to use MIC values in terms mg/cm^2 (Table 5.4.). MIC values of NA against *A. niger* for both films (0.035 mg/cm^2) are more than twice of MIC value for paper discs (0.015 mg/cm^2). The reason for this could be entrapment of active agents with film compounds causing a decrease in antimicrobial activity. Although NA MIC value against *P. roquefortii* in film form was higher (0.0175 mg/cm^2) compared to paper discs (0.015 mg/cm^2) the difference was much lower. This is probably related to susceptibility of *P. roquefortii* to NA as reflected in MIC value also.

WG and MC films containing RE alone did not have any antifungal activity. However, RE had synergistic effect on NA in both films. Although MIC value of NA for WG and MC films was 2 mgNA/10g fs for *A. niger*, zones formed at 1.5 mg NA/10g fs when 1.5 ml RE/10g fs were also added to films (Table 5.2. and 5.3.). However, there were no significant changes in zone diameters at higher NA concentrations as compared to films containing only NA. Therefore, the effect of RE was only apparent at 1.5 mgNA/10g fs for *A. niger*. Also, increasing RE amount to 3 ml did not decrease MIC values and did not increase the zone diameters further for *A. niger*. Synergistic interaction was not observed between NA and RE for *P. roquefortii*.

Table 5.2. Agar disc diffusion results for NA and RE containing WG films

NA conc. (mg/10 g fs)	RE conc. (ml/10 g fs)	Diameter of inhibition zone (cm)	
		A. niger	*P. roquefortii*
0	0	+	+
0.5	0	+	+
1	0	+	2.34±0.15[a]
1.5	0	+	2.72±0.08[b]
2	0	2.76±0.4[a]	3.11±0.11[c]
5	0	3.72±0.18[b]	4.46±0.38[d]
10	0	3.98±0.06[c]	5.23±0.22[e]
20	0	4.01±0.0[c]	5.26±0.20[e]
40	0	4±0.09[c]	5.06±0.11[f]
0.5	1.5	+	+
1	1.5	+	2.5750±0.49[a]
1.5	1.5	1.79±0.11[a]	2.7750±0.21[b]
2	1.5	2.53±0.09[b]	3.2833±0.04[c]
5	1.5	3.58±0.07[c]	4.1583±0.01[d]
10	1.5	3.74±0.11[d]	4.8250±0.01[e]
20	1.5	4.08±0.07[e]	5.4167±0.07[f]
0.5	3	+	+
1	3	+	2.15±0.13[a]
1.5	3	1.71±0.06	2.65±0.18[b]

+ no inhibition, [a-d] same letters show that there is no statistical difference between different NA levels at P>0.05

Table 5.3. Agar disc diffusion results for NA and RE containing MC films

NA conc. (mg/10 g fs)	RE conc. (ml/10 g fs)	Diameter of inhibition zone (cm)	
		A. niger[a]	*P. roquefortii*[b]
0	0	+	+
0.5	0	+	+
1	0	+	2.59±0.09[a]
1.5	0	+	3.04.±0.13[b]
2	0	2.84±0.18[a]	3.37±0.11[c]
5	0	3.51±0.04[b]	4.54±0.35[d]
10	0	3.64±0.2[c]	4.91±0.11[e]
20	0	4±0[d]	4.89±0.13[e]
40	0	3.99±0.11[d]	5.30±0.17[f]
0.5	1.5	+	+
1	1.5	+	2.38±0.14[a]
1.5	1.5	2.29±0.34[a]	2.77±0.27[b]
2	1.5	2.75±0.21[b]	2.93±0.08[b]
5	1.5	3.63±0.14[c]	4.52±0.14[c]
10	1.5	4.09±0.06[d]	5.10±0.19[d]
20	1.5	4.2±0.11[d]	5.46±0.08[e]
0.5	3	+	+
1	3	+	2.39±0.10[a]
1.5	3	1.88±0.05	3.04±0.12[b]

+ no inhibition, [a-d] same letters show that there is no statistical difference between different NA levels at P>0.05

Table 5.4. MIC values for NA, RE and NA+RE in disc and film form

	Disc		WG film		MC film	
Fungus	NA[a]	NA+RE	NA[b]	NA+RE[c]	NA[b]	NA+RE[c]
A. niger	750 (0.015)	Ns	2 (0.035)	1.5+1.5(0.0264+0.0264)	2 (0.035)	1.5+1.5(0.0264+0.0264)
P.roquefortii	750(0.015)	Ns	1(0.0175)	ns	1(0.0175)	ns

pm (mg/cm^2), [b] mg/10g fs (mg/cm^2), [c] mg NA/10g fs+ml RE/10 g fs (mg NA/cm^2 + ml RE /cm^2), ns:no synergy

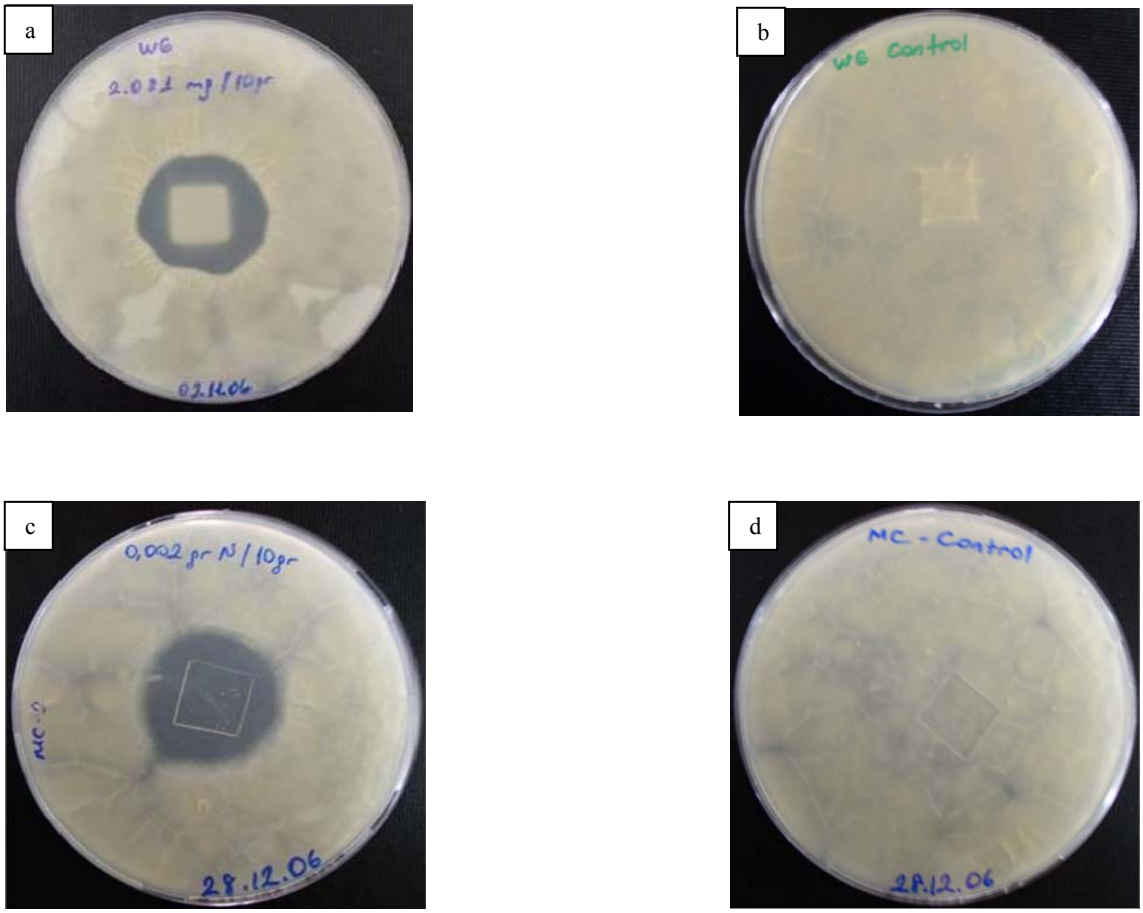


Figure 5.8. Inhibitory effects of WG and MC films against *A.niger* a) WG film containing 2 mgNA/10 g fs b) WG control film c) MC film containing 2 mg NA/10 g fs d) MC control film

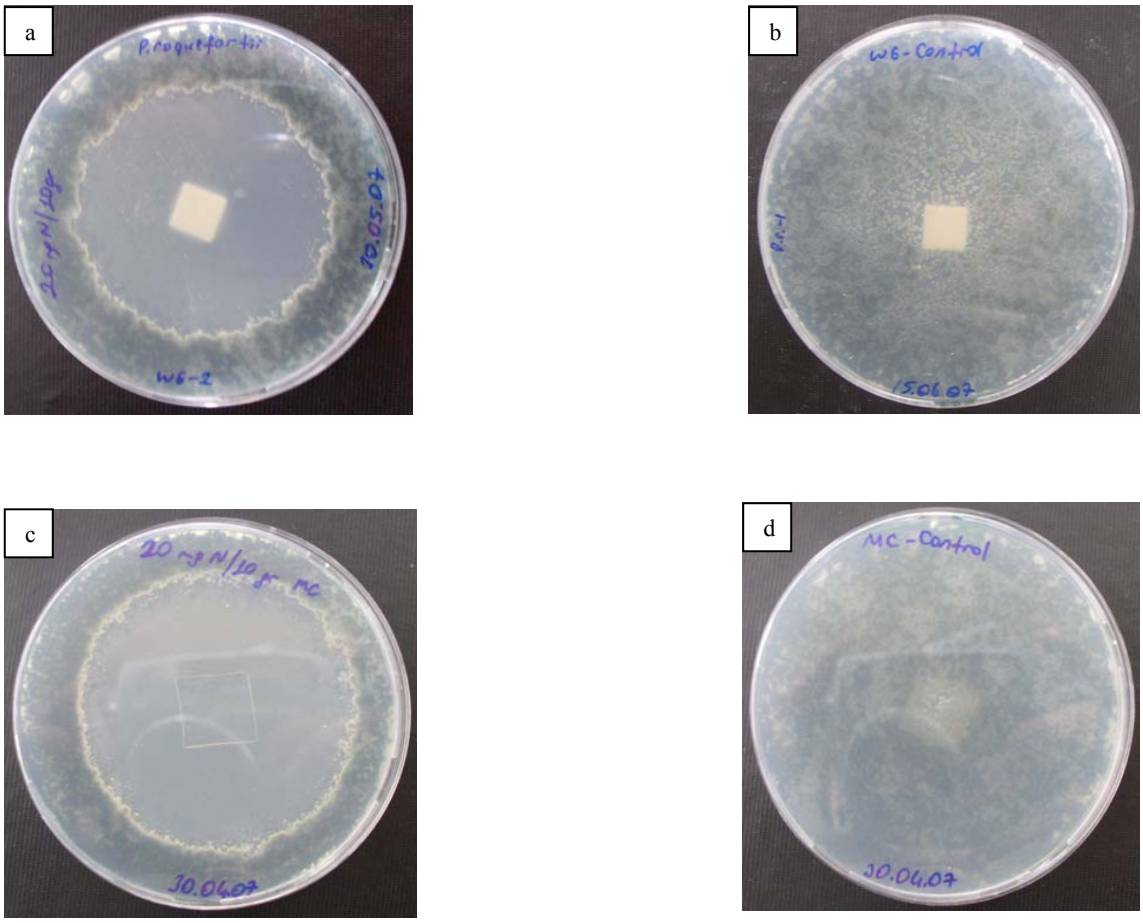


Figure 5.9. Inhibitory effects of WG and MC films against *P.roquefortii* a) WG film containing 20 mgNA/10g fs b) WG control film c) MC film containing 20 mgNA/10g fs d) MC control film

5.5. Mechanical Properties of Antimicrobial Films

TS, EB(%) and EM are commonly examined mechanical properties for evaluating the ability of edible films to protect foods against mechanical abuse (Gennadios 2002). TS is known as the maximum tensile strength that a material can sustain and is taken to be the maximum load on the test specimen during the mechanical testing. EB (%) is usually measured at the point in which the films break and is defined as the percentage of changes of the original length at break. EM can be defined as the ratio of stress to strain in the initial linear part of the stress-strain curves that measures the stiffness of film (Robertson 1993).

WG and MC films prepared in this study were homogenous, thin and flexible. While films prepared from WG were opaque MC films were clear according to visual examination of these films. Adding active agents to films did not have any effect on their visual appearance. Thicknesses of prepared films were presented in Table 5.5. There were no significant differences in thicknesses between control and NA or NA+RE added films except MC containing 20 mg NA/10 g fs.

Mechanical properties of control and NA, NA+RE containing WG and MC films were listed in Table 5.5.

Table 5.5. Mechanical properties of WG and MC films

Film	NA (mg/10gfs)	RE (ml/10gfs)	Thickness (mm)	Mechanical properties		
				TS (MPa)	EM (N/mm^2)	EB%
WG						
	0	0	0.25±0.028^a	2.06±0.27^a	28.79±11.62^a	224.80±53.01^a
	2	0	0.22±0.036^a	2.01±0.28^a	28.21±9.26^a	225.32±49.18^a
	5	0	0.23±0.022^a	2.00±0.17^a	23.29±3.93^a	254.76±56.80^a
	10	0	0.24±0.026^a	2.10±0.19^a	27.25±3.29^a	227.87±41.00^a
	20	0	0.24±0.023^a	2.23±0.11^a	29.62±5.18^a	225.10±20.97^a
	1	1.5	0.23±0.034^a	2.02±0.21^a	20.62±3.44^a	260.92±11.00^a
	1.5	1.5	0.23±0.037^a	2.20±0.09^a	21.19±3.43^a	278.12±16.72^a
	2	1.5	0.23±0.037^a	1.74±0.13^b	14.68±1.08^b	295.95±15.42^b
MC						
	0	0	0.063±0.009^a	36.63±7.53^a	313.23±56.89^{bc}	73.98±13.22^b
	2	0	0.069±0.01^a	37.17±11.95^a	380.73±58.36^a	60.45±17.67^a
	5	0	0.066±0.01^a	33.80±6.11^a	346.03±51.79^{ac}	66.47±6.58^{ab}
	10	0	0.065±0.01^a	27.28±3.80^b	263.87±31.97^b	66.78±2.51^{ab}
	20	0	0.075±0.005^b	22.59±4.98^b	299.90±27.21^{bc}	56.76±9.18^a
	1	1.5	0.064±0.009^a	36.09±5.36^a	426.75±35.47^a	62.20±8.03^a
	1.5	1.5	0.062±0.01^a	32.34±12.48^a	357.80±39.38^b	62.10±16.61^a
	2	1.5	0.066±0.01^a	35.45±13.92^a	341.89±69.43^b	70.46±21.73^a

Incorporation of NA to WG film did not cause major changes in its mechanical properties. Since antimicrobial synergy between NA and RE was observed at low NA concentration, mechanical properties of the films containing 0.5-2 mg NA/10g fs in combination with RE were tested. A decrease in TS (15.5%) and EM (50%) of WG films was observed at 2 mg NA/10g fs+1.5 mL RE/10g fs. In addition, elongation increased with increasing NA concentration for WG films containing RE. Gontard, et al. (1992) produced films at low pH from a solution containing gluten in absolute ethanol, acetic acid, water, and glycerol and indicated that films fabricated at pH 4 had the best mechanical properties. Our data is in agreement with previous studies although the values of this study were slightly different which can be owing to the film formation method, testing conditions and amount of solvents used to produce films. Table 5.6. shows the TS, EB (%) and EM of various wheat gluten based films.

Table 5.6. TS , EB and EM values of various wheat gluten based films

Film type	TS (MPa)	EB%	EM(N/mm^2)	Reference
Wheat Gluten (control)	2.06±0.27	224.80±53.01	28.79±11.62	Present study
Wheat Gluten (control)	2.6±0.2	237.9±21.9	-	Gennadios, et al. 1993
Wheat Gluten (control)	-	213±15	23±2	Olabarrieta, et al. 2006
Wheat Gluten (control)	1.8±0.0	250.5±40.0	-	Kayserilioglu, et al. 2001
Wheat Gluten with γ radiation	2.1±0.5	384±82	29±4	Micard, et al. 2000
Wheat gluten with mineral oil	2.2±0.2	267.2±40.1	-	Gennadios, et al. 1993
Wheat gluten with nisin	1.80±0.6	-	-	Ko, et al 2001

In this study WG films had low TS but high EB when compared to other films (Table 3.4.). It was reported that mechanical properties of WG films depend on processing conditions, types and amount of plasticizers and external conditions such as RH and temperature (Gennadios 2002).

For MC films, TS decreased at 10 and 20 mg NA/10g fs, and reduction in TS at highest NA concentration corresponds to a value of 38.3% relative to control. High concentrations of NA might have been caused a disruption in film matrix resulting in a

change in strength properties of the film. A slight increase in EM of NA containing MC films was observed at low NA concentration. EB (%) values of MC films were also affected by NA incorporation, and a slight decrease was observed. Addition of low concentration of NA in combination with RE did not result any significant changes in TS of MC films. Mechanical properties of MC films were also compared to those found in previous studies (Table 5.7.). As can be seen from Table 5.7. that different MC-Gly ratios and plasticizers used in film preparation strongly affect the film properties.

Table 5.7. TS and EB values of various MC based films

Film type	TS (MPa)	EB(%)	Reference
MC:Gly (1:0.17)	36.63	73.98	Present study
MC:Gly (1:0.5)	25	80	Park, et al. 1993
MC:Gly (1:0.3)	49	37	Donhowe and Fennema 1993
MC:PEG400 (1:0.3)	41	33	Donhowe and Fennema 1993
MC:PEG400 (1:0.44)	19	74	Donhowe and Fennema 1993

As a conclusion, MC films showed considerably higher mechanical properties than WG films. This result is related to the chemical structure of the films. Cellulose based polymers like MC generally consists of long straight chains which exert greater molecular order, therefore resulting in strong and rigid films than protein based films such as WG (Banerjee and Chen 1995).

5.6. FTIR Analysis

IR spectra of control films and films containing antifungal agents at various concentrations were recorded to study the interaction between added agents and the biopolymers (Figure 5.10. and 5.11.) Absorption bands corresponding to N-H stretch at 3280 cm^{-1}, -CH, $-CH_2$ and $–CH_3$ stretching at 2935-2877 cm^{-1}, amide carbonyl group at 1650 cm^{-1}, $-CH_2$ and $–CH_3$ groups at 1445-1415 cm^{-1} and C-N stretch at 1039 cm^{-1} were observed for WG films. MC films had absorbance bands at 3400 cm^{-1} (O-H stretching), 2835-2940 cm^{-1} (C-H stretching), 1645 cm^{-1} (C-O), 1450-1315 cm^{-1} ($-CH_2$ and $–CH_3$ groups) and 1100-1000 cm^{-1} (C-O-C) which are similar to that were reported in

literature (Zaccaron, et al. 2005). All spectra belonging to control films and films containing NA and NA+RE have similar IR absorbance patterns and formation of any new peaks or shifts in the peaks were not observed. Therefore, it could be concluded that there was no interaction between added agents and the WG and MC films. Pranoto et al. (2005) also did not observe any interaction between incorporated antimicrobial agents (garlic oil, potassium sorbate and nisin) and biopolymer, chitosan.

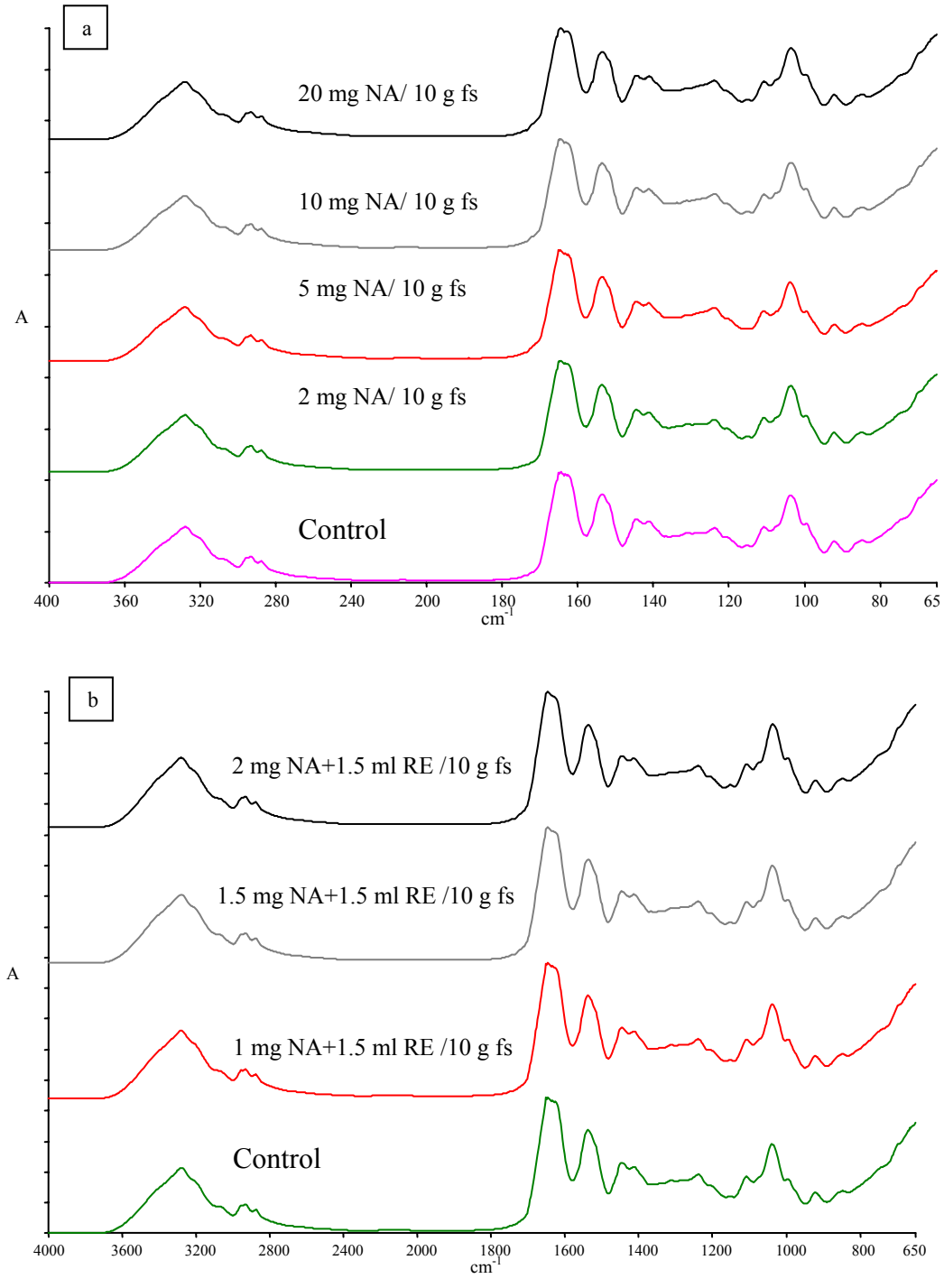
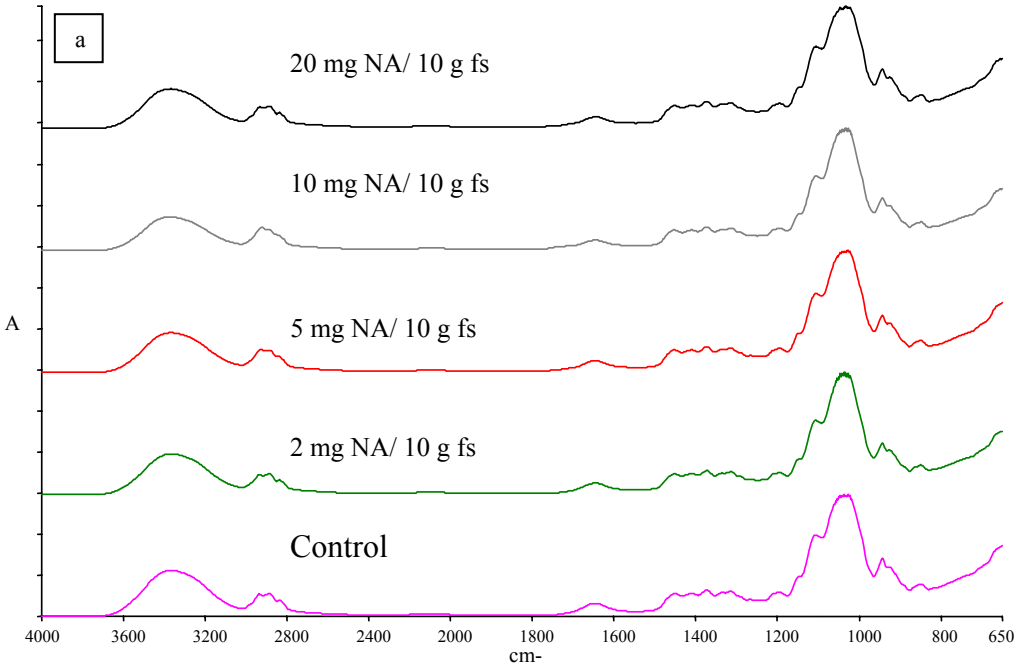


Figure 5.10. FTIR spectra of WG films containing a) NA b) NA+RE



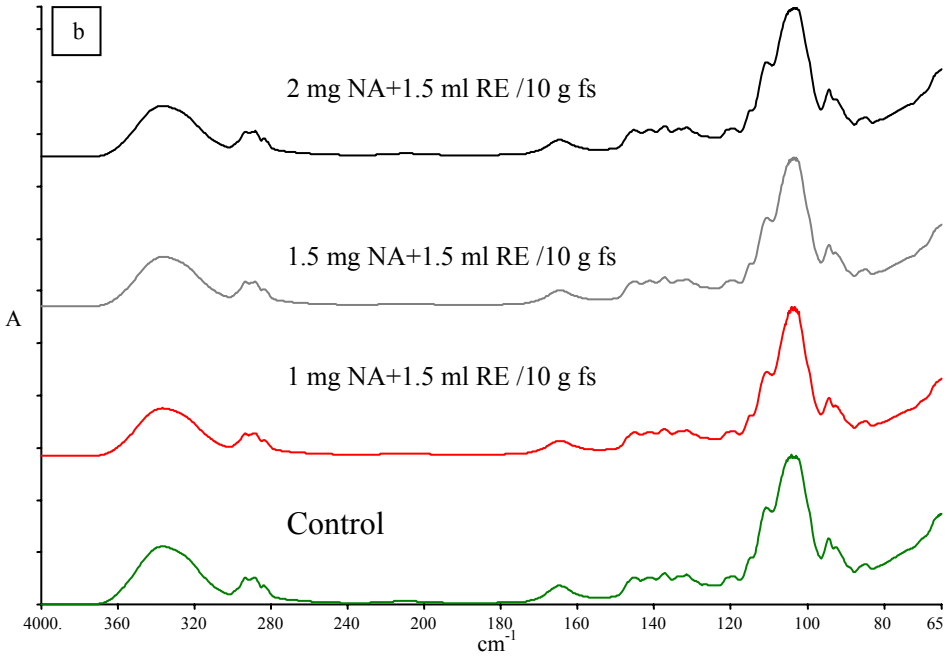


Figure 5.11. FTIR spectra of MC films containing a) NA b) NA+RE

5.7. SEM Analysis

SEM pictures of cross-sections of control films and films containing antifungal agents were also obtained to visually examine the structure of the films. Structure of WG control film was homogeneous as reported by Pochat-Bohatier, Sanchez and Gontard (2006) (Figure 5.12.) There was no visual change in the structure of 2 mg NA/10g fs containing WG film. However, continuity of the film was disturbed at high NA concentrations and holes and dents formed. In addition, NA crystalline structures were observed in the films. It was reported that NA suspension at pH 6.5 has a stable crystalline form (Stark 2003). With the addition of RE (1.5 ml/10g fs) besides to NA into WG films, small road shaped particles were observed in the film (Figure 5.12.). These rod shaped particles are probably RE since films containing only RE had the same particles.

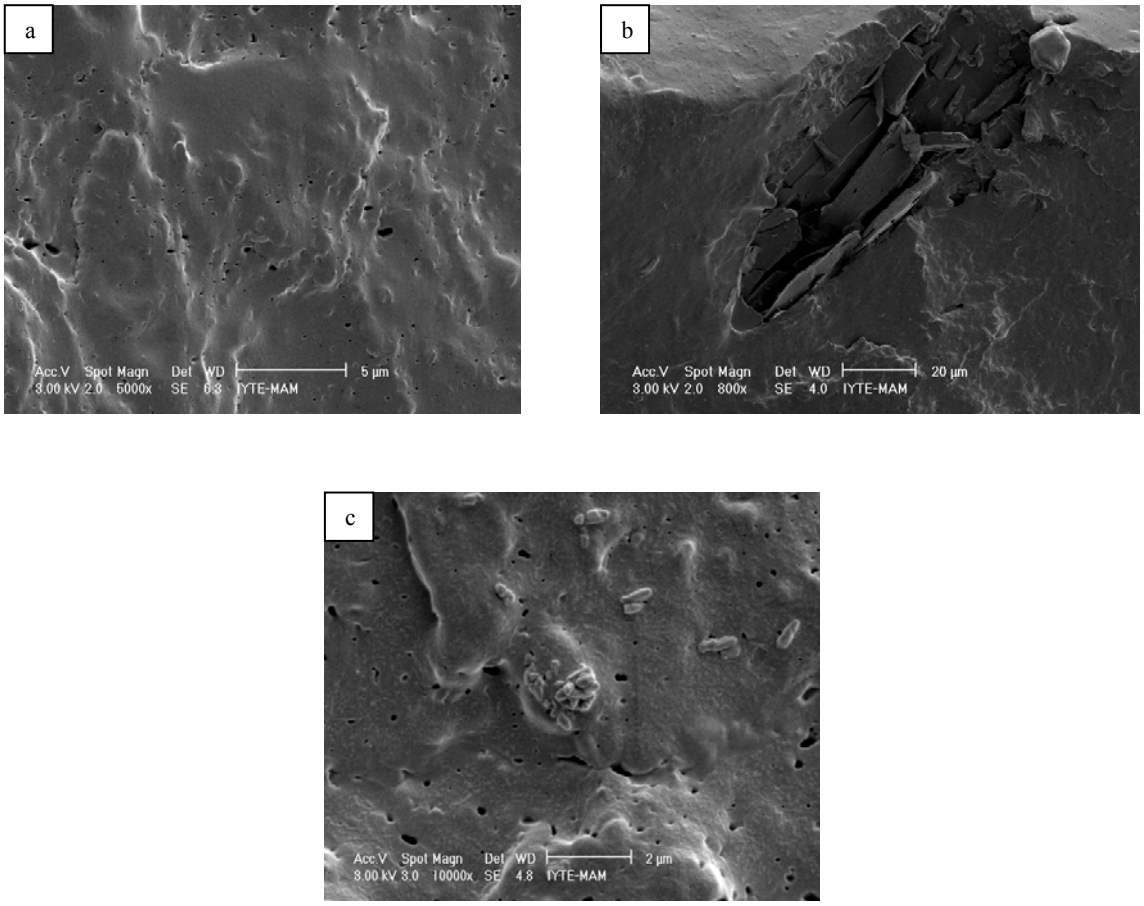


Figure 5.12. SEM pictures of WG films a) control b) 20 mg NA/10g fs c) (2 mg NA +1.5 mlRE)/10g fs

MC control films also had a homogeneous structure (Figure 5.13.). 2 mg NA/10g fs did not cause any change in the structure of MC film. However, at high NA concentrations dents in the film structure were seen. As it was observed in WG films, there were crystalline structures in the film at 20 mg NA/10g fs (Figure 5.13.). Small particles as well as cracks were occurred when RE (1.5 ml/10g fs) was incorporated into MC films in combination with NA.

From SEM observations, it could be concluded that NA homogeneously distributed in WG and MC films at low concentrations while it formed crystals at higher levels.

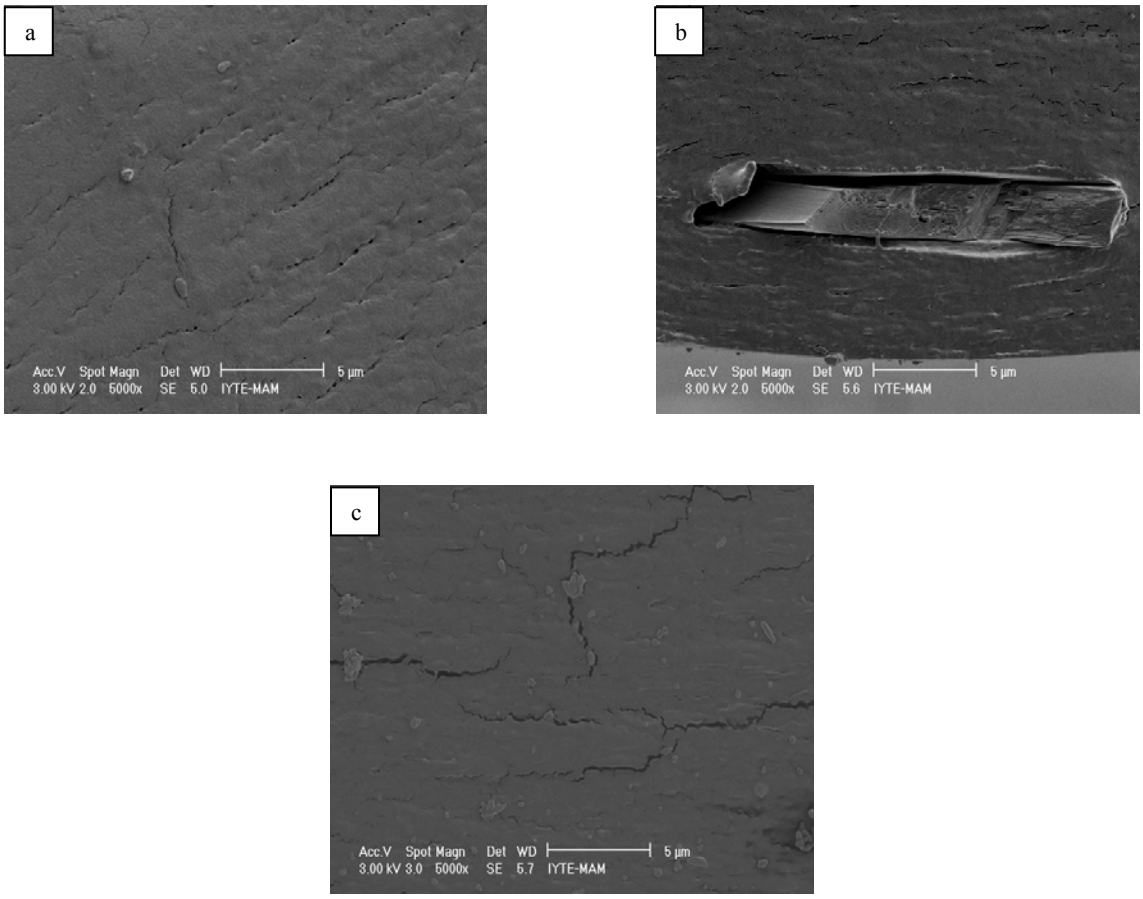


Figure 5.13. SEM pictures of MC films a) control b) 20 mg NA/10g fs c) (2 mg NA +1.5 mlRE)/10g fs

5.8. Water Vapor Barrier Properties of Films

WVP is a measure of the ease with which a material can be penetrated by water vapor (Cagri, et al. 2001). Several types of gravimetric methods based on ASTM E96 (1980) are commonly used to determine the WVP of films (Ko, et al. 2001, Pronato, et al. 2005). It is important to note that this general method is designed to measure the WVP properties of synthetic hydrophobic polymer packaging films and involves numerous assumptions which may not apply to hydrophilic films (McHugh et al. 1993). The first assumption of ASTM method is that structure of the films is accepted as homogenous. However, hydrophilic films such as protein and polysaccharides based films could be non-homogeneous by nature owing to the presence of varying water concentration in the different film section. Secondly, film solubility and diffusivity coefficients are assumed constant. On the other hand hydrophilic films show nonlinear water sorption isotherms and solubility and diffusivity of hydrophilic films which strongly depend on the water content in the film are not constant. It is also assumed that resistance to mass transfer is negligible in gas phases on both sides at the test film. This is also often false because the small distance between the film and the water or desiccant in the cup leads to low levels of natural convention in the cup. Thus, $p_1=p_2$ (Figure 4.2.) assumed by ASTM method is not true for films if adequate fan moving air on the film surface is not maintained (Krochta, et al. 1994, McHugh, et al. 1993). Consequently, measurement of WVP of hydrophilic edible films is very complex, therefore, an alternative method should be used. In our study, WVP correction method was used to eliminate these errors. This method involves calculation of WVTR (Eq: 1), p_2 (Eq: 2), permeance (Eq: 3) and finally WVP of films (Eq: 4) with air circulation which maintains uniform conditions at all test locations.

WVP of control and active agent containing WG and MC films with their thickness and RH estimates at the film underside are provided in Table 5.8. WVP of WG and MC control films were 6.85 and 3.50 g.mm/mkPa.h, respectively. This difference is associated with chemical composition of films because structure and polarity of biopolymers significantly affect the water vapor transport. Linear polymers as in the case of MC can be packaged tightly leading to low permeability However, proteins possessing bulky side chains are loosely packaged and have relatively high permeability (Turhan, et al. 2007). While WG is a hydrophobic material, MC has a

hydrophilic structure. WVTR increases with hydrophilicity and heterogeneity of the polmers (Debeaufort, et al.1997). The RH values at film underside were lower than the assumed 100% RH because of the mass transfer resistance of a stagnant air between the water surface and film (McHugh, et al. 1993). Although Gontard et al. (1993) reported lower WVP values for WG films, results of our study were similar to Pommet et al (2003). Differences could be attributed to film formulations since parameters such as protein concentration and plasticizer amounts could alter WVP values. Differences in WVP measuring conditions also cause differences in WVP values. WVP of MC film was in the range (3.02-4.36 g.mm/mkPa.h) of what was reported by Park, et al (1993). There were no significant differences between WVP of control films and films containing active agents. Therefore, NA and NA+RE incorporation into WG and MC biopolymers did not have any effect on water vapor barrier properties of these films. Active agents did not probably cause any significant structural changes or plasticizing effect in the films. As explained in infrared spectroscopy part there was also no significant interactions between active agents and films. Therefore, WVP values also did not change with addition active agents. Similarly, Rojas-Graü, et al. (2007) reported that adding of essential oils namely, oregano oil, carvacrol, citral and cinnamon oil into the alginate-apple puree edible films did not significantly change their WVP values. Incorporation of nisin into WG films did not cause significant changes on WVP properties of films (Ko, et al. 2001). WG and MC films tested in this study had much higher WVP values than synthetic films. The mean WVP values of low density polyethylene film (LDPE) with the thickness of 0.014mm and Saran® (DowBrands L.P, Indianapolis, IN) having 0.012mm thickness are 0.002 and 0.00016 g mm/m^2.h.kPa at 23±2^{o}C and 55±3 %RH. This considerable difference is attributed to presence of straight chain polymers in synthetic films resulting in a higher degree of molecular order and low permeabilties (Banerjee and Chen 1995). This difference also can be explained by hydrophilic and hydrophobic properties of polymers. LDPE is a hydrophobic polymer, thus, it is expected that solubility of water in that polymer sould be lower than those in either WG or MC film.

Table 5.8. Water vapor barrier properties of WG and MC films

Film	NA (mg/10gfs)	RE (ml/10gfs)	Thickness (mm)	WVP ($g.mm/m^2.kPa.h$)	RH at film underside (%)
WG					
	0	0	0.25±0.028[a]	6.85±0.56[a]	84.29±0.57[a]
	2	0	0.22±0.036[a]	5.99±2.23[a]	83.55±2.99[a]
	5	0	0.23±0.022[a]	6.36±0.67[a]	83.16±0.90[a]
	10	0	0.24±0.026[a]	6.47±0.95[a]	83.49±1.22[a]
	20	0	0.24±0.023[a]	6.42±0.65[a]	83.77±1.33[a]
	1	1.5	0.23±0.034[a]	5.99±0.71[a]	83.95±1.16[a]
	1.5	1.5	0.23±0.037[a]	6.04±0.74[a]	83.90±1.40[a]
	2	1.5	0.23±0.037[a]	6.26±0.25[a]	84.46±1.10[a]
MC					
	0	0	0.063±0.009[a]	3.50±0.25[a]	74.65±1.36[a]
	2	0	0.069±0.01[a]	3.43±0.57[a]	76.35±1.30[a]
	5	0	0.066±0.01[a]	3.20±0.25[a]	74.85±1.26[a]
	10	0	0.065±0.01[a]	3.79±0.41[a]	74.35±2.29[a]
	20	0	0.075±0.005[b]	4.11±0.74[a]	74.33±3.07[a]
	1	1.5	0.064±0.009[a]	3.88±0.23[a]	73.65±1.77[a]
	1.5	1.5	0.062±0.01[a]	3.63±0.59[a]	74.18±2.03[a]
	2	1.5	0.066±0.01[a]	3.65±0.46[a]	74.94±1.83[a]

5.9. Application of MC and WG Films on Kashar Cheese

Post process contamination of foods by undesirable microorganisms especially with molds is one of the most common causes of food spoilage and the substantial economic losses for the food industry. Fungal growth on dairy products such as cheeses also results in health problems owing to mycotoxin production.

In this study, the effects of MC and WG films containing NA and NA+RE on growth of *A. niger* and *P. roquefortii* on Kashar cheese were investigated not only by examining visual mold decay but also enumerating the fungal spores during 30 days storage at 10 ^{o}C. Both films containing NA (2, 5, 10 and 20 mg/10g fs) and MC film

containing NA+RE (2, 3 ,5 mg NA+1.5 ml RE /10g fs) were prepared and cheese slices were wrapped with these films following inoculation with 10^5 spore/ml fungal solution. Table 5.9. shows the effect of various concentrations of NA incorporated into MC films on the growth of *A. niger*.

Table 5.9.Effect of MC films containing NA on the population (log CFU/g) of *A. niger* on Kashar cheese during storage.

Treatment	Day 0	Day 10	Day 20	Day 30
1	0	0	too many to count	
2	0	0	too many to count	
3	3.3±19.75	3.26±3.59	4.28±3.90	5.61±3.11
4	3.3±19.75	3.1±3.20	3.19±3.70	5±7.07
5	0	0	too many to count	
6	3.43±27.5	2.81±1.73	3.13±0.71	4.99±22.73
7	3.54±35	2.51±1.89	3.29±3.53	3.57±4.95
8	3.35±22.5	2.6±1.63	3.3±2.30	3.56±19.40
9	3.34±21.75	2.65±1.29	3.21±0.71	3.63±7.07

1= no treatment, 2= UV treated cheese, 3= *A. niger* inoculated cheese, 4= *A. niger* inoculated +MC control film, 5= notreatment cheese +MC control film, 6= MC film containing 2 mg NA/10g fs, 7= MC film containing 5 mg NA/10g fs, 8= MC film containing 10 mg NA/10g fs, 9= MC film containing 20 mg NA/10g fs

Population of *A. niger* increased from 3.3 to 5.61 cfu/g in inoculated control cheese samples. There was no significant reduction in spore population on cheese packaged with MC film containing 2 mg NA/10g fs compared with control film and *A. niger* inoculated cheese after 30 days of storage at 10 ^{o}C (Table 5.9.) (Figure 5.14.). On the other hand, application of MC films at and above 5 mg NA/10 g fs resulted in nearly 2 log CFU/g reduction of *A. niger* on cheese when compared to control sample (Table 5.9.) (Figure 5.15.). Although MIC of MC films were determined as 2 mg NA/10g fs in *in vitro* studies, this value reached up to 5 mg NA/10g fs in application on cheese. Antimicrobial films or coatings are more effective in terms of inhibiting target microorganisms as applied to nutrient media than on real systems owing to complex structure of foods (Dawason, et al. 2002).

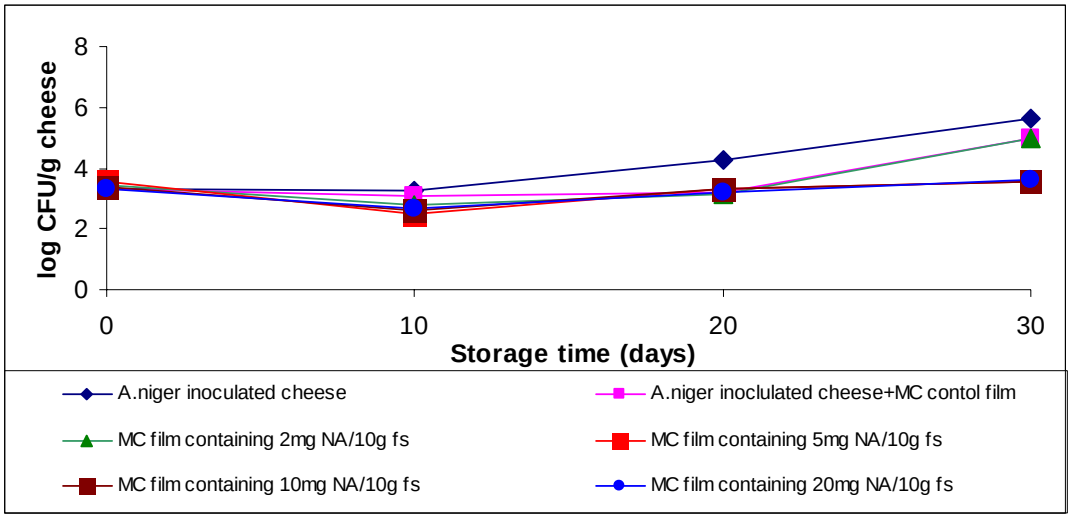


Figure 5.14. Effect of MC films containing NA on the growth of *A. niger* inoculated on Kashar cheese during 30 days of storage at 10°C

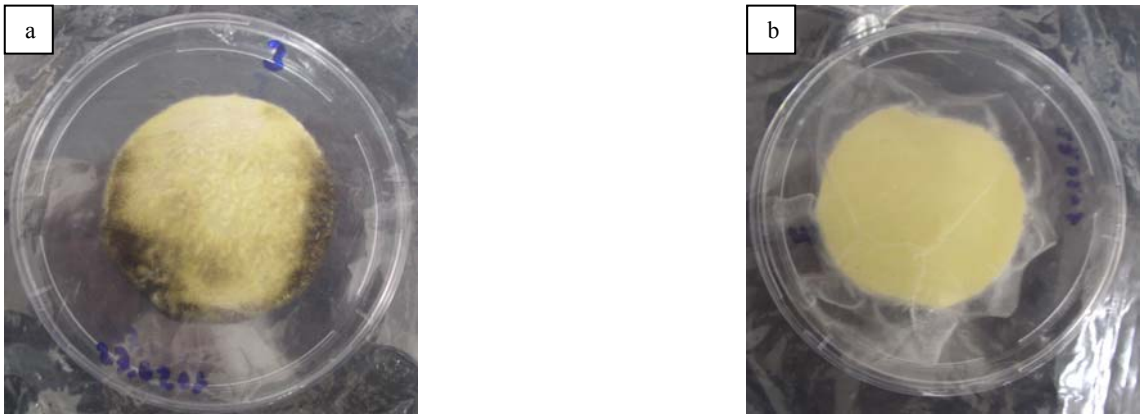


Figure 5.15. Suppression of *A. niger* by MC film a) Contol cheese without any film b) 5 mg NA/10g fs after 30 d storage at 10°C

After 10 days of storage, population of fungus on cheese that was untreated, UV treated and packaged with MC control film increased to levels too many to count (Table 5.8.) However, these types of fungus were not detected on cheese packaged with MC and WG films containing NA during 30 days of storage. In the study of Chen et al. (1999), edible coatings composed of MC including benzoic acid showed inhibitory effect against yeasts on fruits. Inhibitory effect of chitosan-lysozyme based films has been revealed against yeast found on Mozzarella cheese (Duan, et al. 2007). As can be

seen from the Table 5.9. that UV at 254 nm wavelength also did not eliminate the predominant fungus present in control cheese over 30 days storage at refrigerated temperature. This result could be due to parameters of UV application procedure used in our study such as treatment time (10 min for each surface of the cheese), wavelength (254 nm), distance from the UV lamp (15 cm) or surface properties and thickness of sample (1 cm). Jun et al. (2003) investigated the inactivation of *A .niger* spores on corn meal by using pulsed UV light. They found that log reduction of fungal spores was enhanced when treatment time or input voltage increased and when the distance decreased.

As for WG films, logarithmic increase from 4.06 to 7.35 cfu/g was also observed in *A. niger* inoculated cheese starting from 10^{th} day of storage. Growth of molds was completely inhibited in cheese packaged with WG films containing at and above 2 mg NA/10 g fs whereas 4.11 log reductions in mold populations were determined in cheese packaged with WG control films after 30 days of storage (Table 5.10.). MIC of WG films was found as 2 mg NA/10g fs on cheese which was the same as for *in vitro* studies. Duan et al. (2007) also indicated that growth of molds completely inhibited on the Mozzarella cheese packaged with chitosan-lysozyme composite films.

Decrease in mold populations covered with control films in our study could be attributed to a decrease in oxygen permeation into the package. WG films absorbed water from cheese sample and covered the surface of the product very well, thereby, visible mold growth was not observed on cheeses packaged with WG films (Figure 5.16. and 5.17.). Although WG film is insoluble in water it could easily absorb water from foods because of the presence of hydrophilic amino acid residues in its structure and high glycerol content used to prepare the film solution. Thus, water acts as a plasticizer in WG films and increases the free volume of the gluten network. This may lead to release of NA from polymer to surface of cheese samples. Remained NA on food surface, therefore, eliminates the fungal spores during storage. This phenomena created by the water uptake on WG films was also demonstrated in another study (Pochat-Bohatier, et al. 2006). Other inhibitory factors in cheese sample like pH, water activity, or chemical preservatives would also serve to further inhibition of molds (Cagri, et al. 2002).

Table 5.10. Effect of WG film containing NA for the populations (logCFU/g) of *A.niger* on Kashar cheese during storage.

Treatment	Day 0	Day 10	Day 20	Day 30
1	0	0	too many to count	
2	0	0	too many to count	
3	4.06±9.43	3.21±0.96	6.89±47.68	7.35±0.96
4	4.11±5.85	3.56±6.76	3.17±10.87	3.24±0.96
5	0	0	0	0
6	3.99±22.17	3.39±6.65	2.7±4.24	0
7	4.07±9.68	3.52±30.71	2.57±4.35	0
8	3.94±5.91	3.04±0.82	1.4±0.5	0
9	3.97±5.56	2.81±1.91	1.87±0.5	0

1= no treatment, 2= UV treated cheese, 3= *A. niger* inoculated cheese, 4= *A. niger* inoculated +WG control film, 5= no treatment cheese +WG control film, 6= WG film containing 2 mg NA/10g fs, 7= WG film containing 5 mg NA/10g fs, 8= WG film containing 10 mg NA/10g fs, 9= WG film containing 20 mg NA/10g fs

One of the draw backs of edible films obtained from polysaccharides and proteins is that they are sensitive to moisture and these films have poor water vapor barrier properties because of their hydrophilic properties (Cagri, et al. 2002). Both films used in this study absorbed water and resulted in changes in their physical properties. Especially WG films slowly swelled and a marked increase in elasticity was observed visually during 30 days of storage. Wu et al. (2000) also found that WG films swelled when the precooked ground beef patties covered with this film.

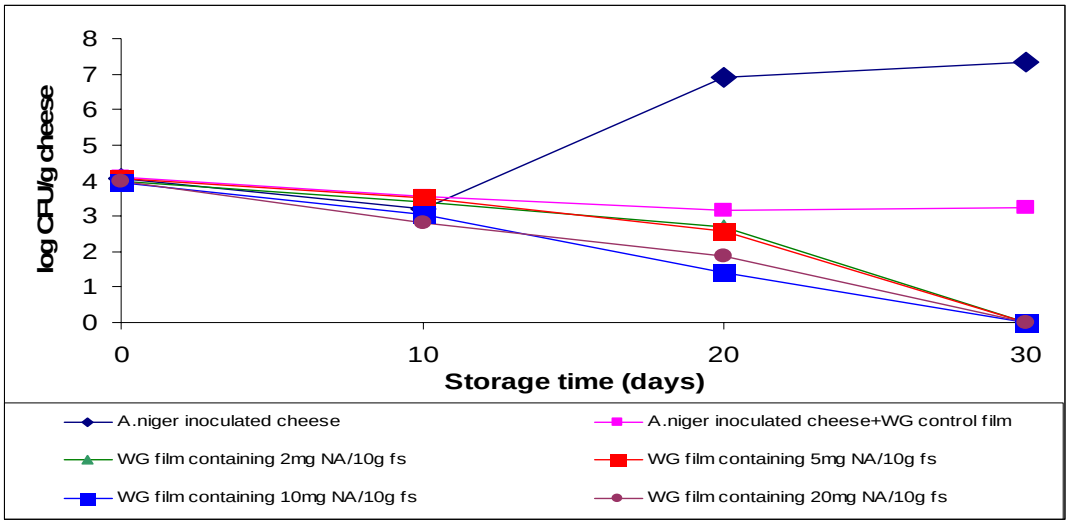


Figure 5.16. Effect of WG films containing NA on the growth of *A. niger* inoculated on Kashar cheese during 30 days of storage at 10°C

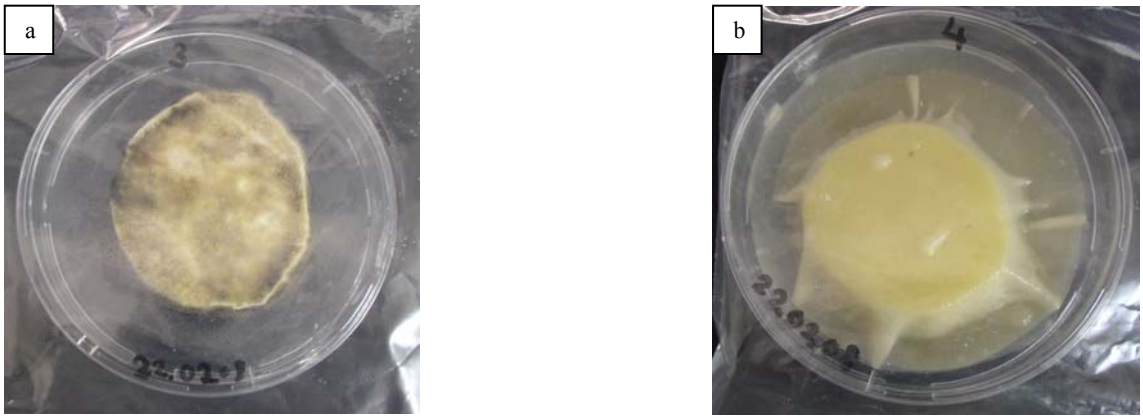


Figure 5.17. Suppression of *A. niger* by WG film a) Control cheese without any film b) WG control film after 30 d storage at 10°C

Synergistic activity of NA+RE incorporated into MC film was also examined on the growth of *A. niger* on Kashar cheese (Table 5.11.). The number of *A. niger* on cheese samples without any MC film and packaged with MC control film increased to 7.73 and 7.46 log CFU/g, respectively after 30 d of storage at 10°C (Table 5.11.). However, the growth of *A. niger* on Kashar cheese was inhibited when MC film containing NA+RE was used (Table 5.11.). Tough there was no significant log reduction between control samples and MC film containing 2 and 3 mg NA+1.5 ml RE,

2.25 log CFU/g reduction was determined between *A. niger* inoculated sample and MC film containing 5mg NA+1.5 ml RE/10g fs (Table 5.11.,Figure 5.18. and Figure 5.19.)

Table 5.11. Effect of MC film containing NA+RE for the populations (logCFU/g) of *A.niger* on Kashar cheese during storage.

Treatment	Day 0	Day 10	Day 20	Day 30
1	0	0	too many to count	
2	0	0	too many to count	
3	3.78±7.87	3.23±7.98	7.5±12.96	7.73±27.94
4	3.76±2.18	3.24±2.86	6.65±10.60	7.46±11.31
5	0	0	too many to count	
6	3.77±11.79	3.3±5.36	5.21±6.36	7.68±28.05
7	3.73±7.63	3.3±4.24	6.92±32.53	7.27±2.52
8	3.78±1.41	3.53±10.35	3.32±14.14	5.48±26.16

1= no treatment, 2= UV treated cheese, 3= *A. niger* inoculated cheese, 4= *A. niger* inoculated +MC control film, 5= no treatment +MC control film, 6= MC film containing 2mg NA+1.5 ml RE/10g fs, 7= MC film containing 3mg NA+1.5 ml RE/10g fs, 8= MC film containing 5mg NA+1.5 ml RE/10g fs

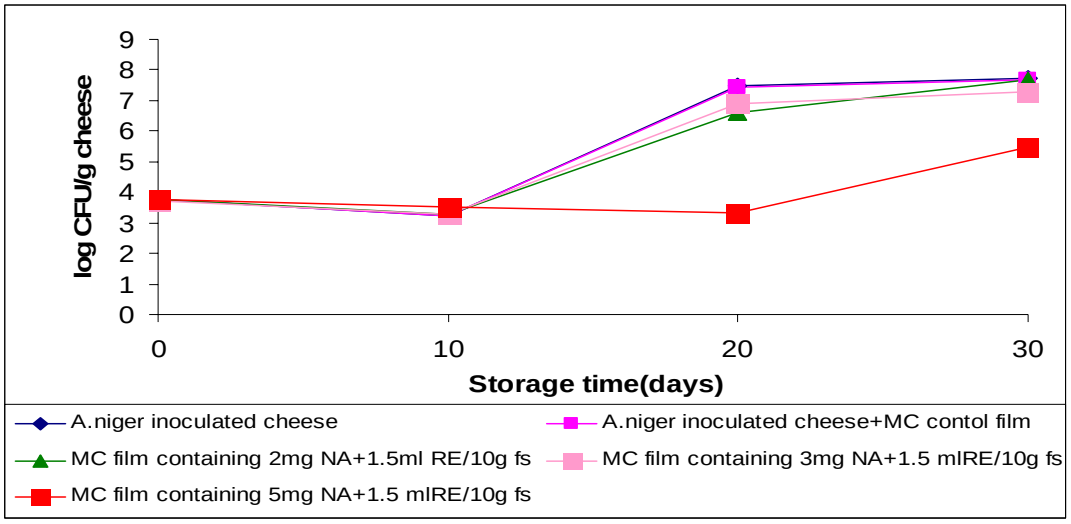


Figure 5.18. Effect of MC films containing NA+RE on the growth of *A. niger* inoculated on Kashar cheese during 30 days of storage at 10°C

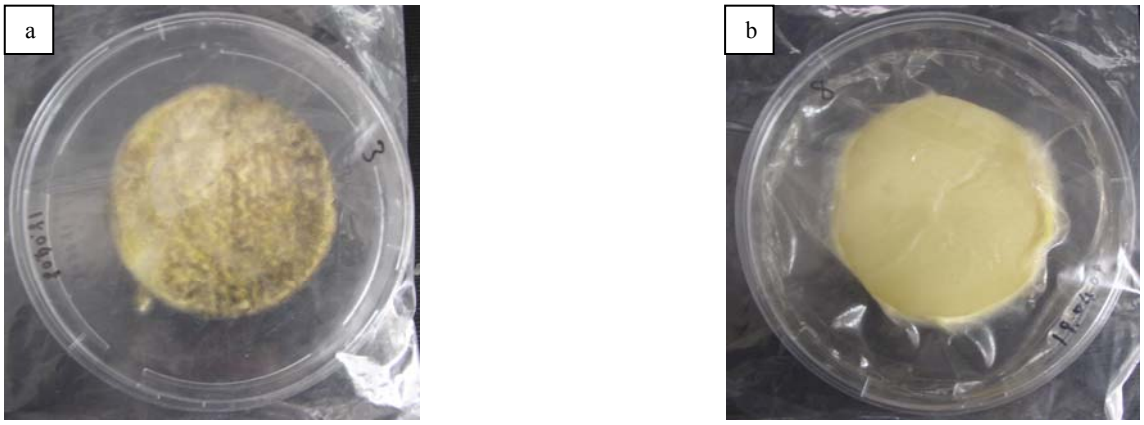


Figure 5.19. Suppression of *A. niger* by MC film a) Control cheese without any film b) MC film containing 5mg NA+1.5 ml RE/10g fs after 30 d storage at 10^{o}C

The antifungal activities of MC and WG film containing NA were also assessed on Kashar cheese using *P. roquefortii* as the microbial contaminant. Table 5.12. shows the antimicrobial effect of MC film containing NA at various concentrations against *P. roquefortii.* Population of *P. roquefortii* inoculated on control cheese samples increased from 2.54 to 7.74 CFU/g after 30 d of storage (Table 5.12.). There was no significant reduction in spore population on cheese packaged with MC control film, MC film containing NA at tested concentrations and *P. roquefortii* inoculated cheese without any MC film after 30 days of storage at 10 ^{o}C (Table 5.12. and Figure 5.20.) Although *P. roquefortii* grows slowly on PDA at 30^{o}C, according to Figure 5.21. the tested mold grew fast and completely covered the cheese slice at 10^{o}C after 30 d of storage. This result indicated that Kashar cheese and refrigeration temperature are suitable environment for *P. roquefortii*.

Table 5.12. Effect of MC films containing NA on the population (log CFU/g) of *P.roquefortii* on Kashar cheese during storage.

Treatment	Day 0	Day 10	Day 20	Day 30
1	0	too many to count	too many to count	
2	0	too many to count	too many to count	
3	2.54±1.48	7.22±24.19	7.25±79.61	7.74±4.39
4	2.6±2.12	7.17±20.32	7.23±41.16	7.84±6.83
5	0	too many to count	too many to count	
6	2.54±1.5	6.76±22.22	6.57±11.61	7.27±7.54
7	2.48±1.22	6.77±25.57	6.60±21.56	7.41±13.62
8	2.68±3.11	6.86±18.00	6.47±46.01	7.49±9.64
9	2.65±1.12	6.87±11.44	6.40±46.49	7.25±5.51
10	2.60±1.87	6.90±6	6.57±1.73	7.40±1.73

1= no treatment, 2= UV treated cheese, 3= *P. roquefortii* inoculated cheese, 4= *P. roquefortii* inoculated +MC control film, 5= no treatment+MC control film, 6= MC film containing 1 mg NA/10g fs, 7= MC film containing 2 mg NA/10g fs, 8= MC film containing 5 mg NA/10g fs, 9= MC film containing 10 mg NA/10g fs, 10= MC film containing 20 mg NA/10g fs

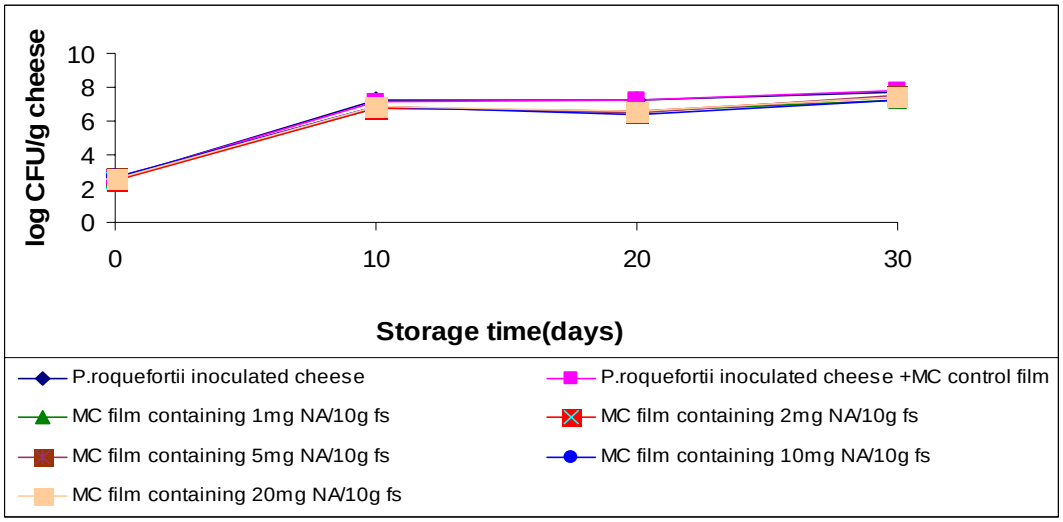


Figure 5.20. Effect of MC films containing NA on the growth of *P. roquefortii* inoculated on Kashar cheese during 30 days of storage at 10°C

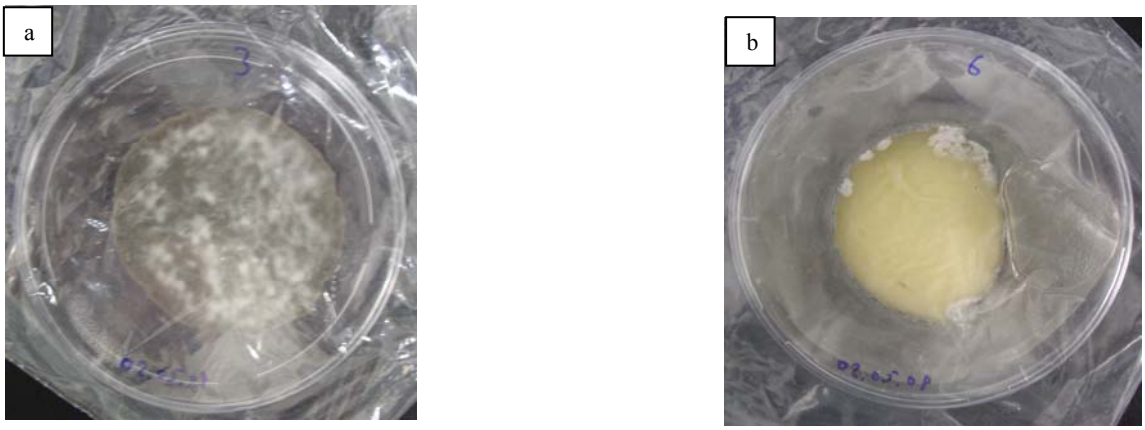


Figure 5.21. Suppression of *P. roquefortii* by MC film a) Control cheese without any film b) MC film containing 5mg NA /10g fs after 30 d storage at 10^{o}C

As for WG films, logarithmic increase from 2.40 to 7.78 cfu/g was also observed in *P. roquefortii* inoculated on Kashar cheese after 30 d of storage.(Table 5.13.) As can be seen from Figure 5.22. that any of the packaging treatment did not significantly cause inhibitory effect when it is compared to control sample. Visible mold incidence (growth) was detected for *P. roquefortii* contaminated cheese samples at the end of storage time as in the case of MC films (Figure 5.23.). However, visible mold incidence was delayed when the inoculated cheese slices were packaged with WG films containing at and above 2 mg NA/10g fs. Mold growth was observed especially on the corner of cheese samples (Figure 5.23.).

Table 5.13. Effect of WG films containing NA on the population (log CFU/g) of *P. roquefortii* on Kashar cheese during storage.

Treatment	Day 0	Day 10	Day 20	Day 30
1	0	too many to count	too many to count	
2	0	too many to count	too many to count	
3	2.40±1	7.14±69.89	7.55±47.09	7.78±5.31
4	2.30±0	7.15±53.80	7.52±89.78	7.61±46.04
5	0	too many to count	too many to count	
6	2.94±2.06	5.47±89.90	7.06±63.07	7.50±58.08
7	2.76±4.5	5.44±4.24	7.25±61.30	7.24±50.11
8	2.54±1.91	5.11±53.28	6.89±26.64	6.99±21.39
9	2.40±1	5.05±76.17	6.91±39.52	6.83±11.42
10	2.51±1.5	5.13±60.16	6.66±27.75	7.05±13.53

1= no treatment, 2= UV treated cheese, 3= *P. roquefortii* inoculated cheese, 4= *P. roquefortii* inoculated +WG control film, 5= no treatment+WG control film, 6= WG film containing 1 mg NA/10g fs, 7= WG film containing 2 mg NA/10g fs, 8= WG film containing 5 mg NA/10g fs, 9= WG film containing 10 mg NA/10g fs, 10= WG film containing 20 mg NA/10g fs

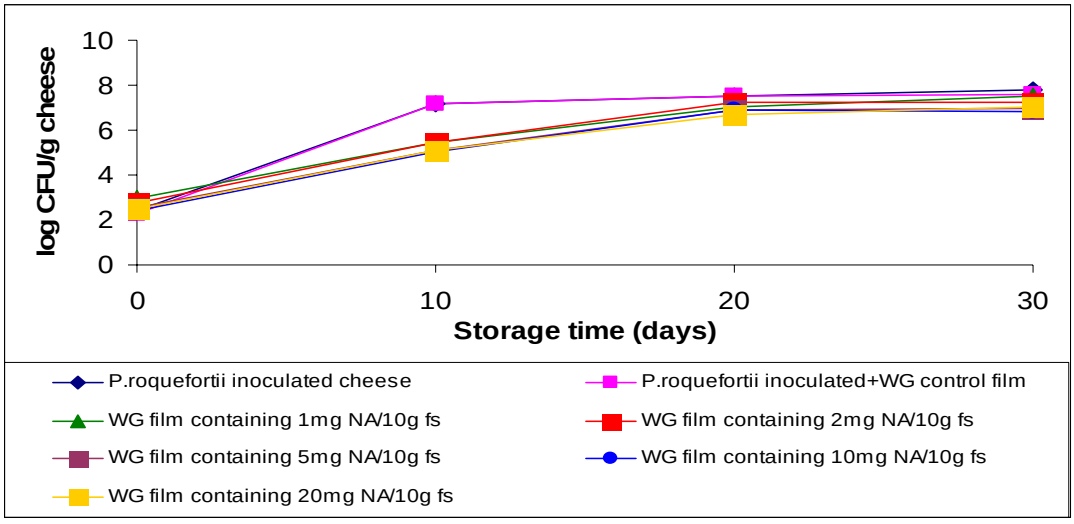


Figure 5.22. Effect of WG films containing NA on the growth of *P. roquefortii* inoculated on Kashar cheese during 30 days of storage at 10°C

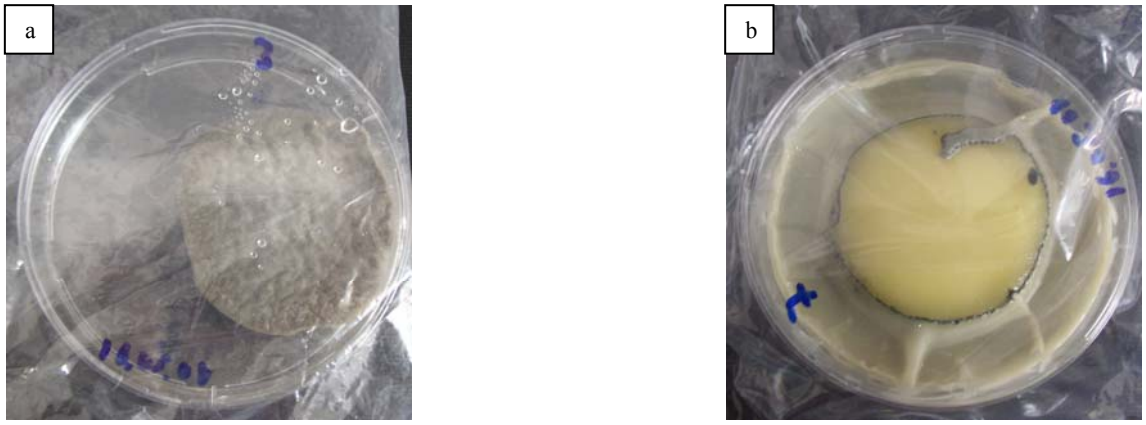


Figure 5.23. Suppression of *P. roquefortii* by WG film a) Control cheese without any film b) WG film containing 5mg NA /10g fs after 30 d storage at 10^{o}C

In the previous studies, antimycotic activity of NA incorporated films has been shown against several molds. Var et al. (2006) reported that packaging materials (PVC, Sperdex-Ref. 99017) and NA used separately prevented mold formation on Kashar cheese ripened for two months and no mold growth was determined because of combined application of these two types of materials and NA during five months ripening period. In the study of Cong et al. (2007) the bilayer coating of chitosan and polyethylene wax microemulsion including NA demonstrated inhibitory effect against two pathogenic fungi known as *Alternaria alternate and Fusarium semitectum* on 20 days storage of Hami melon at ambient temperature. Reps, et al. (2002) used Delvocid containing 50% NA to protect the surface of several types of cheeses such as Gouda and Edam from mold growth. It was reported that polyvinyl acetate including 0.05% NA effectively prevented the growth of undesirable molds on the surface of cheeses (Reps, et al. 2002).

Our results indicated that MC and WG based films incorporated with NA demonstrated antifungal function against *A. niger* inoculated on the surface of Kashar cheese during 30 days storage at 10^{o}C. MC films containing at and above 5 mg NA/10g fs delayed the mold growth and resulted in 1.5 log reduction when compared with control sample. On the other hand, WG films completely inhibited mold at and above 2 mg NA/10g fs during storage. Therefore, both films have potential to be used in prevention and control of toxigenic molds on dairy products such as cheese samples as active packaging materials.

CHAPTER 6

CONCLUSION

In this study, antimicrobial activity of ALF, RE, NA and some of their combinations were tested against several pathogenic bacteria and spoilage molds that cause problems in food products. These active agents were also incorporated into biopolymers prepared from WG and MC. Results from this study indicated that neither ALF nor RE showed inhibitory effect against *L. monocytogenes*, *E. coli* O157:H7, *S. enteritidis*, *A. niger* and *P.roquefortii* when an active agent absorbed paper piece was directly applied on solid media. Same results were also obtained for WG and MC films containing ALF and RE. NA also did not have any antibacterial activity against *L. monocytogenes*, *E. coli* O157H:7 and *S. enteritidis.* On the other hand, NA absorbed paper discs and NA containing WG and MC films displayed very effective antifungal activity against two important spoilage molds that were used in this study. Although RE did not inhibit fungal growth itself, it had synergistic interaction with NA against the growth of *A. niger*. Incorporation of NA to both films had no significant effect on the barrier properties of films but slight changes were observed in tensile properties of MC film containing NA at high concentrations. A decrease in mechanical properties of WG films containing NA in combination with RE also were found. Results of infrared spectroscopic analysis did not show any interaction between films and active agents SEM analysis indicated that NA crystallizes at high concentrations in biopolymers.

Application studies also demonstrated the efficacy of WG and MC films containing NA against *A. niger* inoculated on the surface of Kashar cheese during 30 days storage at 10^{o}C. Given these overall findings, incorporation of NA into biopolymers provides a novel way to enhance the safety and quality of the dairy products.

A final point to be noted is that further studies are required to test the effectiveness of developed films on different foods, microorganisms and contamination levels. Active agents could be also applied on food products in a coating formulation. In addition, release behavior of antimicrobial agents from polymeric matrix needs to be investigated for better understanding of the antimicrobial activity of these active agents.

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