BIOACTIVE FOOD PACKAGING

Strategies, Quality, Safety

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To the memory of Fotis senior,
whose life principles were based on honesty, duty, humility
and endless work.

And to Fotis junior,
who will hopefully follow in his grandfather's footsteps.

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Preface

TRADITIONALLY, food packaging materials were chosen to avoid undesirable interactions with the contained food. Packaging in this case functioned mainly as a protective barrier against the effects of the external environment. During the past twenty years, "active" packaging materials were, and still are being, developed to interact with food, with the objectives of maintaining food quality and/or enhancing the safety of the packaged product. More recently, bioactive food packaging materials have been devised that incorporate biologically active ("bioactive") and functional additives with the expanded objective of exerting a beneficial health effect on the consumer as a result of designed migration of bioactives from the package material into the packaged food.

It is critical that the bioactive substance itself be a natural rather than a synthetic compound and have no adverse effects on the sensory or other properties of food. Obviously, bioactive packaging additives will have to be approved as indirect food ingredients, since upon migration they become food constituents.

The present book focuses on the strategies used for incorporating natural substances into both conventional and biodegradable food packaging materials, including bioactives' applications to the main areas of antioxidant and antimicrobial packaging, with other applications also considered.

This volume is the result of the cooperation of numerous international experts who have already contributed extensively to the literature on the subject of bioactives in packaging. In the first part of the book the various types of bioactive ingredients (oxygen scavengers, en-

zymes, phyto-chemicals, polysaccharides, etc.) that can be impregnated into conventional and biodegradable packaging materials are covered including technologies for their incorporation. A chapter on diffusion/migration of low-molecular-weight compounds from the polymeric matrix into the food phase is also included. The second part of the book investigates specific applications including edible packaging materials, nanotechnology materials, and high-barrier materials. Finally, legislative ramifications of using bioactives in food packaging are briefly covered.

It is our hope this book will assist food and packaging scientists, technologists, students and regulators to understand the concepts and applications of bioactive food packaging and thereby design safer and more functional packaging. We hope also that the book lays the groundwork for future research in what is a new field replete with challenges and opportunities.

MICHAEL G. KONTOMINAS, Ph.D.

CHAPTER 1

Bioactive Packaging of Foods: Quality and Safety Issues

MICHAEL G. KONTOMINAS, Ph.D.

1.1. INTRODUCTION

PACKAGING is without a doubt an integral part of any food production process aiming initially to protect the contained product from environmental factors such as oxygen, light, water vapor, and contamination from airborne microorganisms, but also to protect from mechanical abuse such as shock and vibration. Besides providing protection, packaging interacts with the consumer by serving as a marketing tool and a means of tamper resistance, as well as a source of information on the use of the contained product (Roberson 2006).

Over the past few years, significant advances in packaging science and technology, along with the demand of consumers for more natural, minimally processed foods, has led to the development of active packaging. According to European Union (EU) regulations 1935/2004/EC and 450/2009/EC, active packaging materials are intended to extend the shelf life or to maintain or improve quality and safety of packaged food. This is achieved by the incorporation of components that are either released or that absorb substances into or from the packaged food or the environment surrounding the food.

Active packaging, still under development, involves the use of an-

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tioxidants, antimicrobials, and other naturally occurring and synthetic molecules to achieve its goal (Mexis and Kontominas 2014).

Recently, the concept of functional foods, that is, foods that beyond their inherent nutritional effects, demonstrate to beneficially affect one or more target functions in the human body in a way that is relevant to either the state of well-being and health or to the reduction of the risk of a disease (Rowan 2001), has led to the development of bioactive food packaging, the field of packaging which involves materials or articles that provide a specific health benefit to the host beyond the expected retention of product quality and safety/shelf life extension (Lopez-Rubio *et al.* 2006). It is self-evident that functional substances used as additives in bioactive food packaging should be natural rather than synthetic and that they are approved by national and/or international regulation authorities (Appendini and Hotchkiss 2002).

The recent literature is somewhat confusing regarding the definition of bioactive packaging. It is logical, for instance, that antimicrobial systems including silver-based compounds or triclosan (Coma, 2008) incorporated into conventional packaging materials such as paper, polyethylene (PE), polypropylene (PP), polyvinylchloride, etc., are to be categorized under "active" rather than "bioactive" packaging applications while substances such as essential oils, chitosan, bioflavonoids, etc., known for their antimicrobial, antithrombotic, antioxidant, antiflammatory, cholesterol lowering, and anticancer properties (Kris-Etherton *et al.* 2004), when incorporated into a packaging material, would constitute an application of bioactive packaging.

Several workers (Issepi *et al.* 2008; Appendini and Hotchkiss 1997; Soares and Hotchkiss 1998; Steven 2004) use the term bioactive packaging describing the incorporation of natural antimicrobials (biopreservatives) in conventional polymeric materials such as low density polyethylene (LDPE), polypropylene (PP), etc.

Still, others (Guerra *et al.* 2005) use the term bioactive packaging to imply that the packaging material is biodegradable, i.e., cellophane and the functional additive is of natural origin (nisin).

Thus, the term active packaging may overlap with the term bioactive packaging with regard to the nature of the additive or additives being used and whether or not the additive migrates into the foodstuff.

Based on the above principles, bioactive substances that are suitable for the incorporation into a package wall include phenolic compounds, phytoestrogens, carotenoids, organo-sulfur compounds, plant sterols, monoterpenes, soluble dietary fibers, plant extracts, essential oils, pre-

biotics, bacteriocins, enzymes, probiotics, and marine oils (Lopez-Rubio *et al.* 2006; Juneja *et al.* 2012 ; Kris-Etherton *et al.* 2002).

1.1.1. Phenolic Compounds

Phenolic compounds vary structurally from simple molecules, i.e., phenolic acids with a C6 ring structure, to highly polymerized compounds, i.e., tannins. The flavonoids are the most common polyphenolic compounds present in plant foods. This category of compounds includes flavones, flavonols, isoflavones, flavonones, anthocyanidins, procyanidins, flavan-3-ols, and their glycosides. The vast majority of plant phenolics are simple phenols and flavonoids. Phenolic compounds are abundant in fruits, vegetables, legumes, cereals, olive oil, wine, tea, etc.

Several population studies have reported an inverse association between flavonoid intake and risk of coronary disease and cancer (Yochum *et al.* 1999; Hertog *et al.* 1995). Phenolics, including resveratrol found in wine and grape extracts, have been shown to exhibit antioxidant properties *in vitro*, resulting in reduced susceptibility of platelet aggregation and reduced synthesis of prothrombotic and proinflammatory mediators (Rotondo and de Gaetano 2000). Quercetin is the predominant flavonoid in the diet. Current research suggests a role for quercetin as an antioxidant and anticancer agent (Yang *et al.* 2001). Phenolics in olive oil include hydroxytyrosol, tyrosol, procattechuid acid, syringic acid, vanillic acid, caffeic acid, and p-coumaric acid. High-phenolic olive oil compared to high-oleic sunflower oil reduced low density lipoprotein (LDL) peroxidation in hypercholesterolemic postmenopausal women (Oubina *et al.* 2001).

1.1.2. Phytoestrogens

These are estrogenic compounds including isoflavones, lignans, and coumestans. Structurally they are diphenolic compounds similar to estrogen but act as estrogen antagonists. Isoflavones (genistein and daidzein) are the most extremely studied phytoestrogens. Soy phytoestrogens decrease the extent of atherosclerotic lesion formation in nonhuman primates, reduce LDL oxidative susceptibility in humans, and decrease thrombin formation (Tikkanen *et al.* 1998). Lignans present in flaxseed have been reported to lower LDL cholesterol (Jenkins *et al.* 1999). Evidence is mounting that they may play a significant role in

protection against breast, prostate, and colon cancers (Bingham *et al.* 1998).

1.1.3. Carotenoids

Lycopene is an acyclic carotenoid formed primarily in tomatoes. There is some evidence that Lycopene may have a protective effect against cardiovascular diseases and various forms of cancer (Nguyen and Schwartz 1999). In addition, it reduces LDL oxidative susceptibility *in vitro* (Dugas *et al.* 1998).

1.1.4. Organosulfur Compounds

Such compounds including diallyl sulfide, triallyl sulfide, diallyl disulfide, etc., costituents of garlic oil, have been found to decrease total and LDL cholesterol and triglycerides, exhibit antioxidant activity, and elicit antithrombotic effects decreasing blood pressure (Steiner *et al.* 1996; Gore and Dalen 1994). Diallyl disulfide and diallyl sulfide appear to exert anticarcinogenic effects (Fukushima *et al.* 1997).

1.1.5. Plant Sterols

Phytosterols are present in the nonsaponifiable fraction of plant oils. They include sitosterol, stigmasterol, and campesterol along with stanol/sterol esters. Plant sterols and stanol/sterol esters evoke a significant serum cholesterol-lowering response beyond that attained with a cholesterol-lowering diet (Niinikoshi *et al.* 1997).

1.1.6. B-glucan and Pectin

These are water-soluble dietary fibers occurring in cereals, fruits, and vegetables that have been shown to lower total and LDL cholesterol levels and to reduce the risk of coronary heart disease (CHD) (Brown *et al.* 1999).

1.1.7. Isothiocyanates

These include 2-phenylethyl isothiocyanate, benzyl isothiocyanate, and sulfonophenes occurring in cabbage, cauliflower, Brussels sprouts, and broccoli. They are reported to protect against tumorogenesis in the lungs, breast, stomach, and esophagus (Zhang and Talalay 1994).

1.1.8. Monoterpenes

They are naturally occurring isoprenoids found in essential oils of citrus fruits, cherries, mint, etc. D-limonene and perillyl alcohol have shown efficacy in both cancer prevention and therapy (Crowell 1999).

1.1.9. Prebiotics

Prebiotics are food components that are not digested in the small intestine and enter the colon where they serve as a growth substrate for beneficial intestinal bacteria (Robertfroid 2001). Prebiotics include the nondigestible carbohydrates lactulose and inulin. Inulin and a range of oligosaccharides act as source of fermentable carbohydrate for the beneficial bacteria in the colon. Other biopolymers such as chitosan and some of its derivatives may also exhibit prebiotic characteristics and can be used as micro- or nanofibers or as encapsulating means of other functional additives (Agullo *et al.* 2003).

1.1.10. Enzymes

The objective of immobilizing an enzyme on a packaging material is to catalyze a reaction which is considered beneficial to the host from a nutritional point of view, i.e., decreasing the concentration of an undesirable constituent such as cholesterol by producing a substance beneficial to the health of the consumer. Among the natural substances used for the immobilization of enzymes are carrageenan, chitosan, gelatin, polylactic acid (PLA), alginates, and polyglycolic acid.

Techniques used for the immobilization of enzymes or whole cells include adsorption, ionic binding, covalent attachment, cross-linking, and entrapment/encapsulation (Bakker *et al.* 2000).

A potential enzymic application is the incorporation of b-galactosidase in an UHT-milk container. During storage, b-galactosidase would breakdown lactose to produce a low-lactose or lactose-free product (PIRA 2005).

Methods of incorporation of bioactive substances to packaging materials include:

1. Incorporation of bioactive substances into a sachet included in the package, i.e., fastened to the package wall.
2. Direct incorporation of the bioactive substance into the package wall.

3. Coating of the packaging material with a matrix that serves as a carrier of the bioactive substance.
4. Use of inherently bioactive (antioxidant, antimicrobial) polymers exhibiting film-forming properties, i.e., chitosan or polymers that can be chemically modified to produce bioactive properties (Quattara *et al.* 2000; Coma 2008). Such bioactive polymers can be used *per se* or as part (i.e., a coating) of a conventional packaging material (PE, PP, polyester, or PET, PLA, etc.).
5. Use of bioactive edible coatings directly applied to the food.

In order to incorporate bioactive substances onto or into the packaging material it is obvious that appropriate methods of fabrication are necessary. Parameters to be optimized include time/temperature conditions for mixing of bio(polymers) with the bioactive substance. Of primary importance is also the engineering of the substrate material to attain a reasonably low release rate between the packaging procedure and the consumption of the package contents (controlled release). In case of film fabrication, the limitations of the specific bioactive substance used should be carefully considered, i.e., for substances sensitive to high temperatures, such as certain vitamins, low process temperatures should be used for casting or for the extrusion of materials.

Even though the applications of bioactive food packaging are practically limitless this book will focus on antioxidant and antimicrobial applications. That is, applications in which the active substance will, besides preserving the packaged food, exert a health-promoting effect to the consumer who uses the specific product. However, other applications will be also mentioned.

1.2. ANTIOXIDANT APPLICATIONS OF BIOACTIVE FOOD PACKAGING

Park *et al.* (2012) incorporated the antioxidants thymol, carvacrol, and eugenol into corn zein films which were laminated to linear low density polyethylene (LLDPE). Examination of release kinetics in the gas and liquid phases verified that the antioxidants were effectively released from the films and inhibited oxidation during testing. The films were subsequently used for fresh ground beef packaging and effectively inhibited lipid oxidation while having a positive effect on color stability of beef patties during storage.

Lopez-de-Dicastillo *et al.* (2012a) incorporated ascorbic acid, ferulic acid, quercetin, and green tea extract (GTE) into an ethylene vinyl alcohol (EVOH) copolymer matrix. The efficiency of the films developed was determined in packaging applications involving brined sardines. Monitoring of the peroxide value (PV) and malondialdehyde (MDA) content showed that antioxidant films maintained sardine stability. The maximum protection against lipid oxidation was achieved using the GTE.

Nerin *et al.* (2006) developed a new antioxidant PP film by immobilizing a rosemary extract containing natural antioxidants. The antioxidant properties of the experimental films were tested using both myoglobin and fresh beef steaks. Results showed that, as compared to control PP, the antioxidant films enhanced stability of both myoglobin and fresh meat against oxidation.

In a study carried out by Gemili *et al.* (2010), cellulose acetate (CA) films with different morphological characteristics were prepared by solution casting containing the natural antioxidants L-ascorbic acid and L-tyrosine. Pore size and thus diffusion of antioxidants from the CA film were controlled by adjusting the CA content in the casting solution. Thus, a controlled release of the antioxidants was achieved. The highest antioxidant activity in release test solutions was observed with highly porous L-tyrosine containing films. However, when the porosity of the films was reduced, the antioxidant activity of L-ascorbic acid released in the solution was found to be higher due to trapping of substantial amounts of L-tyrosine in dense films.

Sonkaew *et al.* (2013) evaluated curcumin (CcM) and ascorbyl dipalmitate (ADP) nanoparticles (NPs) incorporated into a cellulose-based film. Macadamia nuts were packaged in a polyamide/polyethylene vacuum pouch containing either the CcMNPs or ADPNPs. Results showed better stability expressed as color retention and PV in nuts packaged in pouches containing the natural antioxidants.

In an ongoing study, Ray and Vakkalanka (2012) incorporated sesamol, a natural antioxidant of sesame seeds, into a series of synthetic films (nylon-6, LDPE, PP, LDPE/PP blend) through extrusion. Results showed that 40–90% of sesamol was retained (depending on extrusion temperature, shear, and polymer morphology) sufficient to impart a substantial antioxidant effect.

In a migration study, Peltzer *et al.* (2013) incorporated carvacrol (1% and 2%) to high density Polyethylene (HDPE) using extrusion. The release of carvacrol was studied in the food simulants: distilled water and virgin olive oil at 25°C and 40°C. The amount of carvacrol migrating

to olive oil was significantly higher than that in water. Experimental results on migration values agreed reasonably well with those obtained by application of a simplified model derived from Fick's second law.

Lopez-de-Diocastillo *et al.* (2010a) incorporated catechin (1% and 5%) and quercetin (0.5% and 2%) as antioxidants in EVOH copolymer film by extrusion. Exposure of the films to different food simulants showed that both compounds were released, migration being dependent on type of food simulant. In aqueous and alcoholic food simulants their release was higher in the case of catechin-containing samples. Extraction in 95% ethanol was also high but negligible in isooctane.

Dopico-Garcia *et al.*, (2010) used the aqueous and methanolic extracts of green tea, black tea, *Lippia citriodora*, and *Hypericum androsaemum* as additives to PP incorporated by extrusion. The antioxidant activity of both extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. Catechin and epicatechin components of green tea proved to be the most effective in providing antioxidant properties to PP as shown by thermogravimetric analysis (TGA).

Lopez-de-Dicastillo *et al.* (2011) incorporated GTE (5%) in EVOH films using extrusion. High performance liquid chromatography (HPLC) analysis of the tea extract compounds revealed their partial degradation during extrusion reducing the content of catechin gallates and increasing the concentration of free gallic acid. In aqueous food simulants in contact with the films, gallic acid was the main antioxidant component released. In 95% ethanol there was a major contribution of catechins. The authors concluded that GTE can be used to develop active/bioactive food packaging materials with antioxidant properties.

In a study by Pereira de Abreu *et al.* (2010), PE films were coated with a phenolic compound extract (7 mg/dm^2 and 24 mg/dm^2) originating from barley husks. The films were used to package frozen Atlantic salmon. Results showed the efficacy of natural antioxidants derived from barley husks to slow down lipid hydrolysis and to increase oxidative stability of salmon flesh.

Domenek *et al.* (2013) extracted lignin (highly branched phenolic macromolecule) from wheat straw and incorporated it into PLA using extrusion and thermo compression. Films were brought in contact with 95% ethanol. The chromatographic study of lignins revealed that the low MW fraction of lignins increased during the polymer processing. Determination of the antioxidant activity of the extract showed that it increased with increasing severity of heat treatment due to the generation of free phenolic monomers during processing.

Nerin *et al.* (2006) incorporated a commercially available natural rosemary extract into a multilayer PP film structure at three different concentrations. The antioxidant properties of the new material were tested by using both myoglobin and fresh beef steaks. Results showed that as compared to the control PP, the films containing the natural antioxidant extract efficiently enhanced the stability of both myoglobin and fresh meat against oxidation.

Bonila Lagos (2013) developed edible films based on chitosan, PLA, PLA and wheat starch (WS) containing thyme or basil and a-tocopherol and citric acid. Experimental films showed a substantial protective effect against oxidation of pork fat.

Lopez-de-Dicastillo *et al.* (2010b) immobilized b-cyclodextrin (10%, 20%, 30%) in EVOH copolymer by using conventional extrusion. The materials with b-cyclodextrin preferentially sorbed apolar compounds such as terpenes. Such films can be used for the binding of undesirable apolar molecules, i.e., cholesterol.

Oussalah *et al.* (2004) prepared protein-based edible films containing oregano, pimento, or oregano-pimento mixtures and applied it to beef muscle slices. The lipid oxidation potential of meat was evaluated by the determination of the thiobarbituric reactive substances (TBARS). Oregano-based films stabilized lipid oxidation in beef muscle samples whereas pimento-based films presented the highest antioxidant activity.

Ponce *et al.* (2008) prepared sodium caseinate, carboxy methyl cellulose, and chitosan edible films containing rosemary, olive, oreganum, capsicum, garlic, onion, and cranberry oleoresins. Minimally processed butternut squash was used as a food substrate, dipped into the film forming solution. Both oleoresins and chitosan enriched with them exerted significant antioxidant activity over polyphenoloxidase throughout storage.

Lopez-de-Dicastillo *et al.* (2012b) prepared EVOH copolymer films containing the flavonoids catechin and quercetin to reduce oxidation of fried peanuts and sunflower oil. Results showed that experimental films reduced the presence of radical oxidation species although the natural antioxidants' migration into the food was limited.

1.3. ANTIMICROBIAL APPLICATIONS OF BIOACTIVE FOOD PACKAGING

Valderrama Solano and de Rojas Gante (2012) incorporated essential

oils (EOs) of oregano and thyme by either coating on a corona treated LDPE film or by extrusion and tested the film against *Listeria monocytogenes*, *Salmonella typhimurium*, and *E. coli* O157:H7. Results showed that films prepared by extrusion (concentration of EOs = 4%) had a higher inhibitory effect than those obtained by ionizing radiation. Films prepared by extrusion (concentration of EOs = 1%) still had antimicrobial properties while those prepared by coating did not.

Oussala *et al.* (2004) studied the antimicrobial properties of whey protein based film containing 1% oregano, 1% pimento or 1% oregano-pimento EOs for the shelf life extension of beef muscle. They showed that films containing oregano EO were the most effective against *E. coli* O157:H7 and *pseudomonas* spp. Films containing oregano extracts showed a 0.95 log reduction of *pseudomonas* spp. and 1.12 log reduction in *E. coli* O157:H7.

Chitosan edible films incorporating garlic oil were compared by Pranoto *et al.* (2005) with conventional food preservatives potassium sorbate and the bacteriosin nisin as antimicrobial agents against *E. coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella thyphimurium*. Garlic oil is composed of sulfur compounds such as allicin, diallyl disulfide, and diallyl trisulfide which possess antimicrobial activity. Results showed that garlic oil incorporated into chitosan films increased the films' antimicrobial activity.

Lee *et al.* (1998) incorporated 1% of grapefruit seed extract (GFSE) in LDPE used for the packaging of curled lettuce. Results showed that the growth of aerobic bacteria and yeasts was reduced. In contrast, 0.1% GFSE yielded no effect on microbial growth in packaged vegetables.

Ha *et al.* (2001) studied GFSE (0.5% and 1%) incorporated into multilayer PE films by both coextrusion and solution-coating and evaluated their antimicrobial activity on ground beef. They found that coating with the aid of a polyamide (PA) binder resulted in a higher level of antimicrobial activity than when incorporated by coextrusion. Both types reduced the growth rates of bacteria in ground beef stored at 3°C as compared to plain LDPE film.

Chung *et al.* (1998) prepared LDPE films impregnated with either 1% *Rheum palmatum* and *Coptis chinensis* extracts and reported that both extracts reduced the growth of TVC, LAB, and yeasts on fresh strawberries. In contrast, An *et al.* (1998) showed that LDPE films containing 1% of the same extracts did not exhibit any antimicrobial activity against *E. coli*, *St. aureus*, *Leuconostoc mesenteroides*, *S. cerevisiae*, *A.niger*, *A.oryzae*, and *Penicillium chrysogenum*.

According to Hang *et al.* (2000) the antimicrobial activity of 5% propolis extract, chitosan biopolymer, or clove extract in LDPE film showed a positive antimicrobial effect against *L. plantarum* and *F. oxysporum*.

Suppakul *et al.* (2002) tested LLDPE films containing 0.05% linalool or methyl carvacrol and reported a positive antimicrobial effect against *E. coli*. In contrast, Chiasson *et al.* (2004) reported no bacteriocidal action of carvacrol against *E. coli* ATCC 25922 in ground beef with the addition of ascorbic acid, a compound with strong antimicrobial properties.

Lim *et al.* (1997) determined the diffusion, solubility, and permeability coefficients of allyl isothiocyanate (AIT) in a Polyvinylidene chloride/polyvinyl chloride (PVDC/PVC) copolymer film in an effort to predict the barrier performance of PVDC/PVC film against AIT vapor. AIT, active ingredient of mustard oil, has been shown to possess a wide pathogen control spectrum (Mari *et al.* 1993) and has been suggested to be used as an antimicrobial vapor in modified atmosphere packaging. Results showed that PVDC/PVC is not a good barrier against AIT vapor.

Allyl isothiocyanate is currently not approved by the United States Food and Drug Administration (USFDA) due to the safety concern that the synthetic compound may be contaminated with traces of the toxic allyl chloride used in the manufacturing process. In Japan allyl isothiocyanate is allowed only in extracts from a natural source (Ishitani 1995).

Scora and Scora (1998) tested a series of mono- and sesquiterpenes for their ability to inhibit growth of three postharvest pathogenic fungi, *Penicillium digitatum*, *P. italicum*, and *P. ulaiense*. Major fungicidal action was observed for phenolic components like carvacrol and related homologue molecules. Monoterpene hydrocarbons gave poor results. Several of the tested compounds may be used as antimicrobial agents in bioactive food packaging applications.

Seydim and Sarikus (2006) prepared edible packaging films made of whey protein isolate incorporating oregano, rosemary, and garlic EOs (1–4%). The films were tested against *E. coli* O157:H7, *L. monocytogenes*, *St. aureus*, *Salmonella enteriditis*, and *Lactobacillus plantarum*. Results showed that films containing the oregano EO were the most effective against microorganisms at 2% concentration. The use of rosemary EO did not show any antimicrobial effect. Likewise, Ponce *et al.* (2008) incorporated 1% of different oleoresins (olive, rosemary, onion, capsicum, cranberry, garlic, oreganim, and a mixture of oreganum plus carvacrol) into chitosan, carboxymethyl cellulose, and casein and tested

their antimicrobial activity against *L. monocytogenes* and natural microflora of squash. Results showed a very limited antimicrobial effect of all oleoresins.

Oussalah *et al.* (2004) prepared milk protein-based edible films containing 1% oregano, pimento, and a 1:1 ratio of oregano-pimento and applied them to beef muscle slices kept at 4°C inoculated with 10^3 cfu/cm^2 of *E. coli* O157:H7 or *Pseudomonas* spp. Results showed that films containing oregano were the most effective against both bacteria whereas films containing the pimento oils were the least effective. A 0.95 and 1.1 log reduction in *Pseudomonas* spp. and *E. coli* O157:H7 respectively was recorded as compared to control samples.

Fernandez-Saiz *et al.* (2009) prepared chitosan films and determined their antimicrobial effect against *St. aureus* and *Salmonella* spp. The work demonstrated the migration of protonated glucosamine fractions from the biopolymer into the microbial culture was responsible for the antimicrobial activity of the biopolymer under studied conditions.

Becerrel *et al.* (2007) tested a patented plastic packaging material containing either a cinnamon extract (active compound trans-cinnamaldehyde) or oregano (active compound carvacrol) against *E. coli* and *St. aureus* using a broth dilution method. Bacterial growth was determined by measuring the optical density at 625 nm. The antimicrobial packaging proved highly effective in controlling the growth of tested bacteria. After extraction from cells, cinnamaldehyde was detected by GC/MS in *E. coli* exposed to the packaging material containing the cinnamon extract.

Joerger *et al.* (2002) coated corona treated ethylene copolymer films with chitosan and tested its antimicrobial activity against *E. coli* and *L. monocytogenes*. Counts of *E. coli* and *L. monocytogenes* were reduced by 5 and 2–3 log respectively after 24 hr. exposure. Tests on beef and chicken meat exudates revealed antimicrobial activity of the film against *E. coli* O157:H7 and *L. monocytogenes* corresponding to a reduction of 2 and 1–2 log cfu respectively. The antimicrobial activity of the film against *L. monocytogenes* was also tested on turkey breast meat resulting in a reduction of 1.7 log after 10 days and 1.2 log after 15 days at 4°C.

The antimicrobial effect of chitosan film incorporating garlic oil was tested against *Salmonella typhimurium*, *L. monocytogenes*, and *Bacillus cereus* by Pranoto *et al.* (2005). Incorporation of garlic oil up to at least 100 µl/g were found to have antimicrobial activity against all three pathogens. Garlic oil components did not affect the physical and mechanical properties of chitosan films.

Tortak and Nizanhoglu (2011) tested the antimicrobial efficacy of PP films coated with chitosan solution (2%) and chitosan solutions containing essential oils (oregano and clove oils) against *L. monocytogenes* and *E. coli* O157:H7 inoculated on to Kasher cheese slices stored at 4°C for 14 days. Results showed antimicrobial effectiveness of all types against the two pathogens with films containing oregano oil showing the greatest antimicrobial effect. Results suggest that chitosan is an ideal biopolymer for coating PP films and EOs have the potential to be used as antimicrobial coatings for biopolymers used in packaging.

Bonilla Lagos (2013) prepared biodegradable films made of WS, polyvinyl alcohol and polylactic acid by incorporating EOs of thyme and basil, a-tocopherol, and citric acid. Results showed that the antimicrobial activity of EOs were enhanced when combined with chitosan in minced pork.

Del Rosario Moreira *et al.* (2011) prepared chitosan (CH), sodium caseinate (SC), and SC/CH films by the casting method and tested their antimicrobial activity against the native microflora of cheese, salami, and carrots. SC did not have any antimicrobial effect on native microflora of foodstuffs tested. Both CH and SC/CH coatings exhibited a significant bacteriocidal activity against mesophilic, psychrophilic, yeasts, and mold count (2–4.5 log cfu/g reduction).

Duan *et al.* (2007) investigated the antimicrobial activity of chitosan-lysozyme composite films and coatings against *L. monocytogenes*, *E. coli*, *Pseudomonas fluorescens*, molds, and yeasts inoculated into Mozzarella cheese slices vacuum packaged and stored at 10°C up to 14 days for bacteria and up to 30 days for fungi. Chitosan treated cheeses showed 0.4–1.3, 0.4–1.4, and 0.3–1.4 log reductions in *E.coli*, *P. fluorescens*, and *L. monocytogenes*, respectively. Incorporation of lysozyme in chitosan showed a greater antimicrobial effect than chitosan alone. Mold and yeasts increased to 10^3 cfu/g in untreated cheese after 30 days. Growth of molds was completely inhibited in cheese packaged with chitosan/lysozyme films. All chitosan/lysozyme packaging applications resulted in 0.01–0.64 log reduction on yeasts populations.

Coma *et al.*, (2002) prepared chitosan edible films by the solution casting method (1% chitosan in aqueous acid solution) and tested their antimicrobial activity against *L. monocytogenes* inoculated on the surface of agar plates. No inhibition zone was obtained from chitosan films deposited on agar medium inoculated with Listeria strains after 24 h of incubation. Emmental cheese slices were subsequently coated with chitosan and incubated at 37°C for 36 hr. Results showed that *L. in-*

nocua counts were reduced by 1 log in the chitosan treated cheeses samples as compared to the chitosan-free samples. After 84 hr. (3.5 days) of storage at 37°C, no colonies of *L. innocua* were detected in chitosan treated samples. In a similar study, Zivanovich *et al.* (2005) prepared chitosan films (1% chitosan in 1% acetic acid aqueous solution) incorporating oregano essential oil at concentrations of 1% and 2%. Films were placed between slices of bologna inoculated with 10^4, 10^5, 10^6, and 10^7 cfu/ml of bacterial suspension (*L. monocytogenes* and *E. coli* O157:H7). Samples were stored at 10°C for 5 days. Pure chitosan films resulted in a 2 log reduction in *L. monocytogenes* counts in bologna samples whereas films with 1% and 2% oregano EO decreased *L. monocytogenes* counts by 3.6–4 logs and *E. coli* O157:H7 by 3 logs.

Devlieghere *et al.* (2004) used a chitosan solution to coat strawberries and lettuce inoculated with a culture of *Candida lambica* and stored at 7°C. Mold growth was determined by weighing the strawberries with visible mycelium growth. On day 12 of storage, 19% of chitosan treated strawberries were rejected as compared to 49% for untreated strawberries. With regard to lettuce, results showed an immediate decontamination activity of chitosan which, however, disappeared after 4 days (no difference in microbial counts between treated and untreated samples). In another study carried out by Coma *et al.* (2003), a 5% chitosan solution was tested for its antimicrobial activity against *Pseudomonas aeruginosa* inoculated at 10^3 and 10^4 cfu/g into Emmental cheese. After incubation (5 days), cheese samples were homogenized in a stomacher in 50 ml of sterile saline solution. Colony counting was performed after inoculation of 1 ml of supernatant solution on the surface of a solid agar medium. Results showed that cheese samples stored for 6 days at 37°C exhibited a higher number (> 300 cfu/petri dish) after 2 days of incubation of petri dishes compared to low number of colonies (< 5 cfu/petri dish) in chitosan coated samples.

GFSE was incorporated (0.5% or 1%) on the food-contact surface of multilayered PE film by either coextrusion or solution-coating process (Ha *et al.* 2001). Films were tested for their antimicrobial activity in ground meat samples stored at 3°C. The film coextruded with 1% GFSE showed antimicrobial activity only against *Micrococcus flavus* while the film coated with 1% GFSE showed activity against *E. coli*, *St. aureus*, and *B. subtilis*. Both types of GFSE-incorporated multilayer PE films contributed to a reduction of growth rates of aerobic and coliform bacteria on ground meat as compared to untreated PE film. More recently, Fernandez-Saiz *et al.* (2008) used renewable blends of gliadin

and chitosan ranging from 0% to 100% for each component to prepare films from solution casting. Films were tested for their antimicrobial activity against *St. aureus*. Results showed a significant inhibitory effect on both pure and composite films on cell viability of *St. aureus*. Antimicrobial activity increased by increasing the amount of chitosan in the film formulation. Furthermore, the amount of chitosan released into the medium was quantified using the Ninhydrin test. Antimicrobial test results correlated well with the amount of chitosan released from the film.

Moller *et al.* (2004) prepared chitosan-hydroxy propyl methyl cellulose (HPMC) films by casting from solution and tested them against *L. monocytogenes* using an agar plate method. Petri dishes were inoculated with 300 cells of *L. monocytogenes* while experimental films were deposited on the surface of the inoculated tryptose agar. Plates were incubated at 30°C. Results showed that films made of 1% chitosan, 0.5% chitosan-1.5% HPMC, 1% chitosan-1.5% HPMC, and 1.5% chitosan-1.5% HPMC exhibited a very high antilisterial activity.

Quattara *et al.* (2000) studied the effect of the incorporation of acetic or propionic acids into a chitosan matrix with or without the addition of lauric acid or cinnamaldehyde applied to bologna, cooked ham, and pastrami. Results showed that propionic acid was nearly completely released from the chitosan matrix within 48 hr. of application whereas release of acetic acid was more limited with 2–22% of acid remaining in chitosan after 168 hr. of storage. Addition of lauric acid, but not cinnamaldehyde, to the chitosan matrix generally reduced the release of acetic acid significantly. Release was more limited onto bologna than the other two meat products. Antibacterial activity of films was tested against indigenous LAB, Enterobacteriaceae, *L. Sakei*, and *Serratia liquifaciens* surface inoculated onto the meat products. The growth of Enterobacteriaceae and *S. liquefaciencs* was delayed or completely inhibited as a result of film application whereas LAB were not affected by the antimicrobial films.

Vartinainen *et al.* (2005) immobilized chitosan onto plasma activated biaxially oriented polypropylene (BOPP) using glutaldehyde as a cross linking agent. Films had strong antimicrobial activity against *B. subtilis* and *E. coli* as determined using the antimicrobial test. Reduction was 4.5 log for *B. subtilis* and 3 log for *E. coli*. Finally, Hu *et al.* (2002) worked with PET films prepared by irradiation of polyester, grafting with acrylic acid and N-vinyl formamide. Through hydrolysis with acid, amide groups were converted to amino groups. Chitosan was then grafted to these fibers via esterification or imine formation. Antibacte-

CHAPTER 2

Bioactive Agents and Polymers

PANUWAT SUPPAKUL

2.1. INTRODUCTION

THIS chapter introduces in a general and brief manner the subjects discussed across the book. Thanks to the recent publication of the European Commission regulation on active and intelligent materials, and to the increasing number of submissions to the U.S. Food and Drug Administration, bioactive packaging will continue to draw attention both from academia and industry for implementation in the area of plastic packaging materials. To date, a plethora of bioactive packaging materials have been reported and reviewed (Cha and Chinan 2004; López-Rubio *et al.* 2006; Coma 2008; Balasubramanian *et al.* 2009; de Azeredo 2009; Espitia *et al.* 2012; Khan *et al.* 2014), all designed and proposed to prevent a broad range of undesirable phenomena, including microbial proliferation, rancidity, gas buildup, color loss/change, nutrient loss, flavor loss, and insect infestation. The focal point of this chapter is to provide an overview of bioactive agents that have the potential to enhance the preservation of packaged products in the distribution chain and, upon migration into the food, to exert a health benefit effect to the host (Lagaron 2005; López-Rubio *et al.* 2006) with a particular emphasis on emerging polymer-based materials.

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2.2. FUNCTIONS OF BIOACTIVE AGENT

2.2.1. Inhibition of Microorganisms

Prevention of pathogenic and spoilage microorganism growth in foods is usually achieved by using chemical preservatives acting as an antimicrobial (AM). However, the recently increasing demand for minimally-processed foods, the need for shelf-life extension of foods, as well as the potential toxicity, carcinogenicity, and teratogenicity of numerous chemical preservatives have led food manufacturers to seek alternative preservation means (Conner 1993; Nychas 1995). A final reason to seek natural antimicrobials for food preservation is the growing concern of microbial resistance towards conventional preservatives (Schuenzel and Harrison 2002).

2.2.2. Inhibition of Oxidation

Oxidative deterioration of fat components in food products is responsible for off-flavors and rancidity which decrease nutritional and sensory quality. The addition of an antioxidant (AO) blocks the oxidative chain reactions of oxygen with unsaturated fatty acids resulting in the preservation of product quality. Synthetic antioxidants (e.g., butylate hydroxytoluene [BHT], butylate hydroxyanisole [BHA], tert-butylhydroxyhydroquinone [TBHQ], and propyl gallate [PG]) are widely used as antioxidants in the food industry. Their safety, however, has been questioned. BHA has been shown to be carcinogenic in animal experiments (NTP 2014). At high doses, BHT may cause internal and external hemorrhage, which leads to death in some strains of mice and guinea pigs (Ito *et al.* 1986). Thus, there is considerable interest among food manufacturers to use natural antioxidants as a replacement for synthetic antioxidants currently used (Plumb *et al.* 1996).

2.2.3. Oxygen Scavenging

The presence of oxygen in food packages may facilitate (1) the growth of aerobic bacteria, yeast, and molds, (2) the development of off-flavors and off-odors, (3) the discoloration and oxidation of labile pigments, and (4) the loss of nutritive values, thereby causing a substantial decrease in the shelf life of foods (López-Rubio *et al.* 2008). Consequently, the control of oxygen levels in food packages is crucial

to either minimize or limit the rate of these deteriorative and spoilage reactions in foods. In addition to the reaction of oxygen with unsaturated fatty acids several moist ready-to-eat (MREs) food items are subject to nonenzymatic browning reactions. This type of degradation is facilitated by the presence of oxygen within the package and may be reduced by packaging food items in oxygen scavenging packaging materials. The most common oxygen scavengers are based on iron oxidation. Although iron-based oxygen scavengers (usually contained within a sachet placed inside the package) are effective and widely used, several aspects, such as accidental ingestion, leak from the sachet, problem with microwave oven in the kitchen, and metal detector in the production line may impair their applications. These drawbacks could be solved by an alternative enzyme-based oxygen scavenging system.

2.2.4. Conversion of Sugars

Lactose intolerance, also called lactase deficiency, is the inability to digest lactose, a sugar found in milk and, to a lesser extent, milk-derived dairy products. It is not a disorder as such, but a genetically-determined characteristic. Lactose intolerant individuals have insufficient levels of lactase, an enzyme that catalyzes hydrolysis of lactose into glucose and galactose, in their digestive system. In most cases this causes symptoms which may include abdominal bloating and cramps, flatulence, diarrhea, nausea, borborygmi (rumbling stomach), or vomiting (Vesa *et al.* 2000). Most mammals normally cease to produce lactase, becoming lactose intolerant, after weaning (Swallow 2003), but some human populations have developed lactase persistence, in which lactase production continues into adulthood. It is estimated that 75% of adults worldwide show some decrease in lactase activity during adulthood (Pribila *et al.* 2000). The frequency of decreased lactase activity ranges from 5% in northern Europe through 71% for Sicily to more than 90% in some African and Asian countries (Bulhões *et al.* 2007). Apart from fresh milk, fermented milk products (e.g., yoghurt, fermented cheese) are fairly well tolerated by lactose intolerant people because lactose is hydrolyzed (predigested) by the microbial lactase present in these products (Schaafsma 2008). However, lactase production by lactic acid bacteria in raw milk may be limited as milk is kept at refrigeration temperature (Claeys *et al.* 2013). To overcome this problem arising from the lack of lactase, an incorporation of lactase into the packaging material for lactose conversion has been introduced (Brody and Budny 1995).

2.2.5. Removal of Cholesterol

As a lipid component, cholesterol is a sterol and an essential structural component of animal cell membranes that is required to establish proper membrane permeability and fluidity for life. Each cell synthesizes it from simpler molecules, a complex 37-step process that starts with the intracellular protein enzyme HMG-CoA reductase. However, normal and particularly high levels of fats (including cholesterol) in the blood circulation, depending on how they are transported within lipoproteins, are strongly associated with the progression of atherosclerosis. Therefore, all food packages in the United States must be labeled regarding cholesterol content for consumer awareness (Brody and Budny 1995).

2.2.6. Suppression of Enzymatic Browning

Appearance and color are key attributes to consumers when selecting and purchasing fresh foods. However, many fresh food items (e.g., fruit and shrimp) contain enzymes that will produce "browning" of the food over a relatively short period of time. This color change not exclusively adversely affects the visual appeal of food items, but negatively affects flavor and nutritional value as well. For instance, fresh-cut fruit processing operations can induce undesirable changes in product color and appearance during storage and marketing. The phenomenon is usually caused by the enzyme polyphenol oxidase (PPO), which in presence of oxygen, converts phenolic compounds into dark colored pigments (melanins) (see Figure 2.1) (Zawistowski *et al.* 1991). Sulfites such as sodium sulfite, sodium bisulfite, sodium metabisulfite, and potassium metabisulfite are widely used as antibrowning agents in the food in-

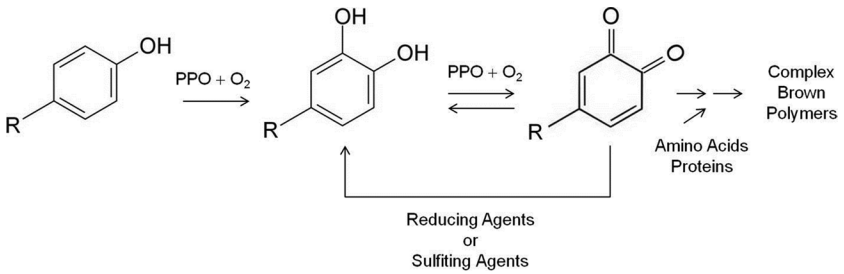

FIGURE 2.1. *Simplified diagram of the enzymic browning initiation by polyphenol oxidase (PPO). Adapted from Walker (1977).*

dustry. A number of products used to prevent enzymatic browning can cause problems themselves as well. These can create severe allergic reactions and may even be life threatening. In addition to episodic and acute symptoms, sulfites may also contribute to chronic skin and respiratory symptoms (Vally *et al.* 2009). For these reasons, alternative compounds that can be used in either food preparation or food packaging are required.

2.2.7. Juice Debittering

A major problem in the citrus industry worldwide is the formation of bitterness in some early- to midseason citrus juice products within hours of extraction from the fruit or, if heated, within a few minutes. The problem occurs in a variety of oranges (including tangerines), grapefruit, and lemons. Washington navel, Satsuma, Natsudaidai, and Shamouti oranges are particularly prone to this problem (Prakash *et al.* 2002; Fayoux *et al.* 2007). Naringin is common in bitter citrus species such as pomelo, grapefruit, sour orange, and pomelo hybrid Natsudaidai (Hasegawa *et al.* 1996). Naringin is high in young tissues and decreases upon maturation. This flavonoid is an indigenous component in both membrane and albedo of the fruit and contributes to the bitterness of fresh fruit and juice. Its taste threshold is approximately 20–50 ppm (by HPLC) (Hasegawa *et al.* 1995; Berhow 2000). In commercial grapefruit juice production, the enzyme naringinase is used to remove the bitterness created by naringin.

2.2.8. Nutrient Fortification

Edible coatings and films have been claimed as effective carriers of many functional ingredients, such as antimicrobial agents to improve safety and stability of foods, antioxidants to prevent lipid oxidation, and flavorings and pigments to improve the sensory quality of food (Kester and Fennema 1986; Rojas-Graü *et al.* 2009; Falguera *et al.* 2011). Along with increased market demands on nutritionally fortified foods, edible coatings and films containing high concentrations of dietary supplements would provide an alternative to food fortification that otherwise cannot be achieved with common processing approaches. This would be especially advantageous for unprocessed or fabricated foods such as fresh or minimally processed fruits and vegetables. Products may be either coated or wrapped with nutritionally fortified coatings

or films. Fortification of dietary biopolymer-based packaging materials with bioactive compounds is one of the most challenging technologies in the field of edible films and coatings (Park and Zhao 2004; Wang *et al.* 2012). A clear understanding of the interactions between the film matrix and the nutraceuticals is essential for developing such edible coatings and films. Along the same line of reasoning, functional additives exerting a beneficial effect on host's health may be incorporated into the packaging material (Lagaron 2005; López-Rubio *et al.* 2006).

2.2.9. Aroma Release

When people eat and drink, first they smell the aroma released from the product that provides an anticipation of the flavor they are about to experience. People smell their food to determine freshness and to gauge if they will like what they are about to eat. Next, as foods and beverages enter the mouth they release vapors that travel up through the retro nasal canal, past the nasal passages, until they reach the olfactory bulb where they are translated as flavor by the brain. Aromas are a powerful marketing tool triggering nostalgic memories which ultimately lead to decision making of purchasing. Fragrances for plastics are used in a variety of applications and are playing an emerging role in marketing food and beverage packaging and in consumer products for the home (Markarian 2006). The incorporation of aromas into the polymer material can be employed to draw consumers' attention when the package is opened and also to balance any detrimental impacts of aroma loss (Koontz 2006). An aroma- and flavor-releasing technology for packaging, developed by US company ScentSational Technologies, is being tested in consumer trials for bottled water in the United States (O'Sullivan and Kerry 2008).

2.2.10. Insect Repellence

Annual post-harvest losses resulting from insect damage, microbial deterioration, and other factors are estimated to be 10–25% of production worldwide (Matthews 1993). Insects are a problem in stored grain throughout the world because they reduce the quantity and quality of grain (Sinha and Watters 1985; Madrid *et al.* 1990). *Sitophilus* and *Tribolium* species are major pests of stored grains and grain products in the tropics (Howe 1965). Control of these insects relies heavily on the use of synthetic insecticides and fumigants, which has led to problems such

as contamination of the environment, increasing costs of application, pest recovery, pest resistance to pesticides, and lethal effects on nontarget organisms, in addition to direct toxicity to users. Thus, repellents, fumigants, and insecticides of natural origin are rational alternatives to synthetic insecticides (Jilani and Saxena 1990; Jembere *et al.* 1995; Okonkwo and Okoye 1996).

Although finished products can be shipped from production facilities uninfested, insects can enter packaged goods during transportation or storage in the warehouse and retail stores. Ultimately, the consumer of the products holds the manufacturer responsible for any insect infestation, even if the cause of the problem is poor storage by a third party. Packaging is the last line of defense for processors against insect infestation. *Sitophilus* spp., *Rhyzopertha dominica* (F.), *Plodia interpunctella* (Hübner), *Lasioderma serricorne* (F.), and *Stegobium paniceum* (L.) are some of the stored-product insects that are capable of penetrating food packaging. However, *Tribolium* spp., *Cryptolestes ferrugineus* (Stephens), and *Oryzaephilus* spp. cannot penetrate intact packages and must enter through existing holes in the package (Highland 1991; Chung *et al.* 2011).

2.3. SOURCES OF BIOACTIVE AGENT

2.3.1. Essential Oils

Recently, essential oils (EOs) have been extensively applied to food, pharmaceuticals, cosmetics, and animal feed due to a perceived higher risk of synthetic materials to the consumer (Burt 2004; Brenes and Roura 2010; Bajpai *et al.* 2012; Solórzano-Santos and Miranda-Novales 2012; Jayasena and Jo 2013). EOs, which are aromatic and volatile oily extracts obtained from aromatic and medicinal plant materials including flowers, buds, roots, bark, and leaves (Hyldgaard *et al.* 2012) by means of expression, fermentation, extraction, or steam distillation (Burt 2004), are one of the best such alternatives to synthetic additives, given their strong biological activities (Zhang *et al.* 2006; Jayasena and Jo 2013). EO constituents may contain color, odor, and/or flavor which yield unique characteristics to foodstuffs. These vary with species, parts of plant, plant age, and local environment (Tragoolpua 1996). Examples of herbs and spices are bay (*Pimenta racemosa*), betel (*Piper betel*), black mustard (*Brassica nigra*), brown mustard (*Brassica juncea*), cin-

namon (*Cinnamomum iners*), clove (*Syzygium aromaticum*), curcumin (*Curcuma longa*), eucalyptus (*Eucalyptus polybractea*), fingerroot (*Boesenbergia pandurata*), galangal (*Alpinia galangal* L.), garlic (*Allium sativum*), ginger (*Zingiberis rhizome*), green tea (*Camellia sinensis*), holy basil (*Ocimum sanctum*), lemongrass (*Cymbopogon citratus*), neem (*Azadirachta indica*), oregano (*Origanum vulgare*), rosemary (*Rosemarinus officinalis*), sage (*Salvia officinalis*), sweet basil (*Ocimum basilicum*), thyme (*Thymus vulgaris*), wasabi (*Wasabia japonica*), etc. Examples of some chemical compounds found in EOs are shown in Table 2.1. Chemical structures of selected constituents of essential oils are also shown in Figure 2.2.

2.3.1.1. Betel Oil

Betel is a tropical plant closely related to the common pepper and belongs to Piperaceae family. Atal *et al.* (1975) reported that betel oil contains chavicol, allylpyrocatechol, chavibetol, methyl chavicol, methyl eugenol, 1,8-cineole, eugenol, caryophyllene, and cadinene. In addition, Rimando (1986) reported that betel oil also contains chavibetol acetate, allylpyrocatechol diacetate, carvacrol, campene, methyl chavibetol, eugenol, pinene, limonene, safrole, and allylpyrocatechol monoacetate.

Bhattacharya *et al.* (2006) have found that betel ethanolic extract appears to be a promising formulation for further investigation as a new natural photo-protector. Several researchers have reported that betel extract and betel oil showed AM and AO activities in model systems (Salleh *et al.* 2002; Lei *et al.* 2003; Dilokkunanant *et al.* 2004; Suliantari *et al.* 2005; Bhattacharya *et al.* 2006). EO of betel showed AM activity against nine pathogenic and spoilage bacteria including *Aeromonas hydrophila*, *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Escherichia coli* O1587:H7, *Listeria monocytogenes*, *Micrococcus luteus*, *Salmonella Enteritidis*, and *Staphylococcus aureus* and three strains of yeast including *Candida albicans*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces rouxii* using an agar well diffusion assay. Using an agar dilution method ranged from 0.78 to 200 μL mL^{-1}, the minimum inhibitory concentrations (MICs) of betel oil in a range of 12.5–100 μL mL^{-1} could inhibit the growth of all test microorganisms (Suppakul *et al.* 2006a). This oil also exhibited AO activity against oxidative bleaching of β-carotene using a β-carotene agar well diffusion assay. The minimum oxidative bleaching inhibitory concentration (MOBIC) of betel oil was 100 μL mL^{-1} (Suppakul *et al.* 2006a).

TABLE 2.1. Chemical Compounds Found in Essential Oils.

Class	Subclass	Structure	Substructure	Examples
Terpenes	Monoterpenes	Acyclic		Citronellol, geraniol, linalool, β-myrcene, β-ocimene
		Cyclic	Monocyclic	Carvacrol, *p*-cymene, limonene, piperitone, pulegone, α-terpinene, β-terpinene, γ-terpinene, terpinolene, thymol
			Dicyclic	Ascaridole, bornane, camphene, carane, 1,8-cineole, fenchane, pinane, α-pinene, β-pinen, thujane
			Tricyclic	Pinene oxide, tricyclene
		Irregular		Artemisia ketone, chrysanthemol, lavandulol, nezukone, α-thujaplicin, β-thujaplicin (hinokitiol), γ-thujaplicin
	Sesquiterpenes	Acyclic		Nerolidol, farnesene, farnesol
		Cyclic	Monocyclic	Abscisic acid, α-bisabolene, α-bisabolol, β-bisabolol
			Dicyclic	β-caryophyllene, chamazulene, eudesmol, guaiol, widdrol
			Tricyclic	Cedrene, santalol
	Diterpenes	Acyclic		Phytol, plaunotol
		Cyclic	Monocyclic	Camphorene (dimyrcene)
			Dicyclic	Clerodanes, labdanes
			Tricyclic	Phyllocladene, 16-kaurene
			Tetracyclic	Jatrophodione, prionitin
			Pentacyclic	Cafestol
	Norterpenes			β-damascone, β-ionone
Phenylpropanoids				Anethole, chavibetol, chavicol, cinnamaldehyde, dillapiole, eugenol, hydroxycinnamic acid, methyl chavibetol, methyl chavicol (estragole), methyl eugenol, myristicin
Sulfur and nitrogen compounds				Allicin, allyl isothiocyanate, methyl anthranilate, mint sulfide, pyridines, pyrazines

Adapted from Suppakul *et al.* (2003), Bakkali *et al.* (2008), Carson and Hammer (2011), Bajpai *et al.* (2012), and Jayasena and Jo (2013).

2.3.1.2. Cinnamon Oil

Cinnamon is a member of the Lauraceae family and is traditionally harvested in Asian countries. It is, perhaps, one of the oldest herbal medicines, having been mentioned in Chinese texts as long as 4,000 years ago. Cinnamon oil has exhibited health beneficial properties, such as AM activity. Cinnamon oil contains cinnamaldehyde, ethyl cinnamate, eugenol, β-caryophyllene, linalool, and methyl chavicol (Hu *et al.* 1985; Chang *et al.* 2001).

Cinnamon oil exhibits antifungal, antiviral, antibacterial, and larvi-

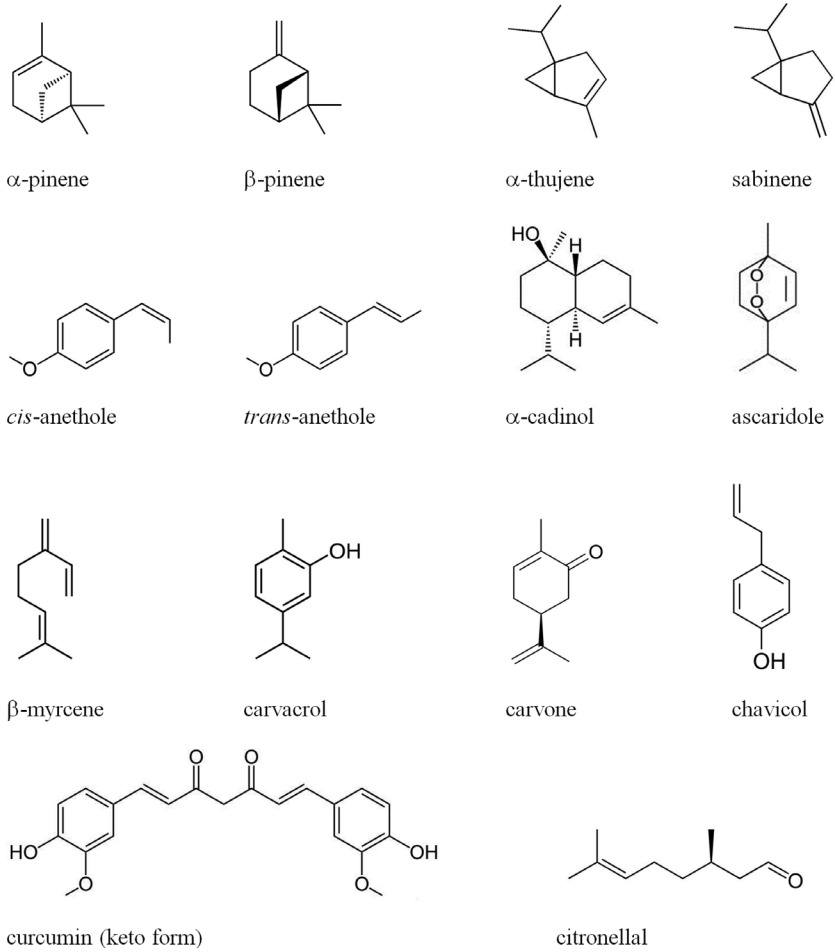

FIGURE 2.2. *Chemical structures of selected constituents of essential oils.*

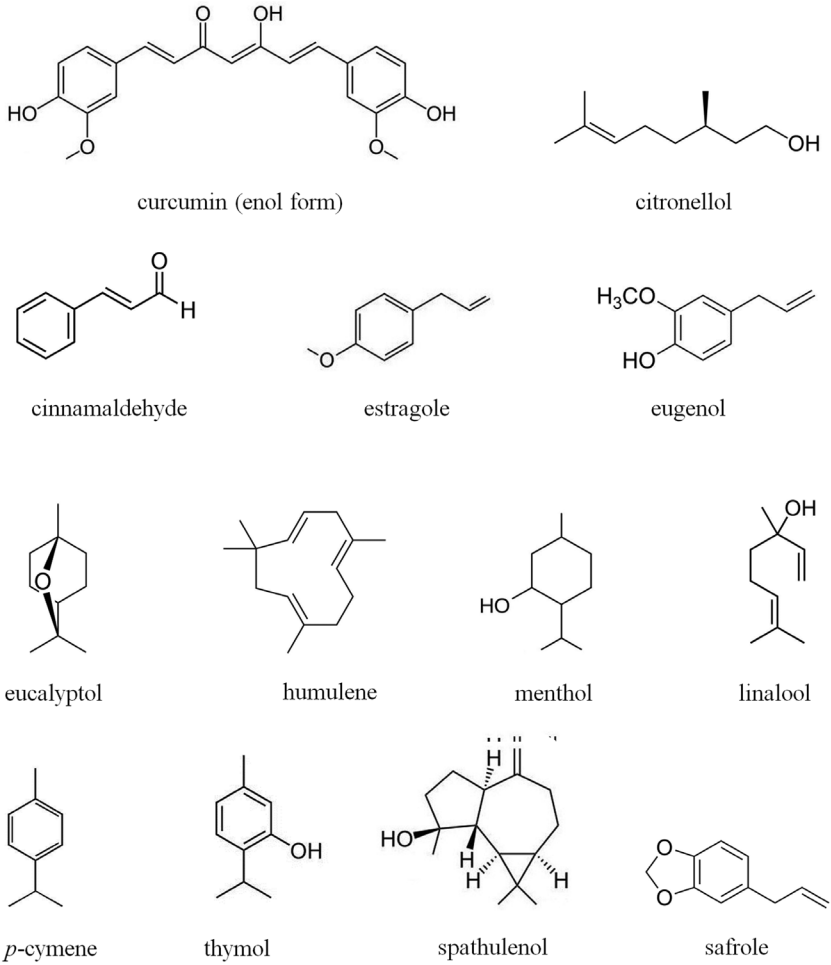

FIGURE 2.2 (continued). *Chemical structures of selected constituents of essential oils.*

cidal activities. Specifically, constituents in cinnamon are able to kill *E. coli*, *C. albicans*, and *S. aureus*. In addition, cinnamon oil's AM and antifungal properties have also drawn considerable attention from many researchers (Hili *et al.* 1997; Ouattara *et al.* 1997; Chang *et al.* 2001; Kim *et al.* 2004). Cinnamaldehyde, which was identified in the oil, is an effective inhibitor of the growth of yeasts, bacteria, and molds as well as toxins production by micro-organisms. It can completely inhibit the growth of a number of bacteria such as *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., and *Enterobacter* spp. (Masuda *et al.* 1998). The MICs of cinnamon oil and cinnamaldehyde in the range of 6.25–25

and 0.78–12.5 μL mL^{-1}, respectively, could inhibit the growth of *A. hydrophila*, *B. cereus*, *E. faecalis*, *E. coli*, *E. coli* O1587:H7, *L. monocytogenes*, *M. luteus*, *S. Enteritidis*, *S. aureus*, *C. albicans*, *S. cerevisiae*, and *Z. rouxii* (Sanla-Ead *et al.* 2012). This oil exhibited AO activity against oxidative bleaching of β-carotene using a β-carotene agar well diffusion assay and radical scavenging activity against free radicals using a 2,2-diphenyl-1-picryhydrazyl (DPPH) assay. The MOBIC of cinnamon oil was 50 μL mL^{-1}. At a concentration of 0.39 μL mL^{-1} solution in ethanol, cinnamon oil yielded the radical scavenging activity of 91.13% (Phoopuritham 2007; Phoopuritham *et al.* 2012) with the half maximal scavenging concentration (SC_{50}) of 0.0197 μL mL^{-1} (Phoopuritham *et al.* 2012).

2.3.1.3. Clove Oil

Clove belongs to the Myrtaceae family. Clove is indigenous to the Moluccas and is widely cultivated in Madagascar, Sri Lanka, Indonesia, and the south of China. Clove oil has biological activities, such as antibacterial, antifungal, insecticidal, and AO properties. Clove oil consists of major phenolic compounds: eugenol, caryophyllene, and eugenyl acetate (Pallado *et al.* 1997). Eugenol acts as AO (Dorman *et al.* 2000) and as AM (Farag *et al.* 1989; Blaszyk and Holley 1998). Eugenol showed an inhibitory effect on the growth of *L. monocytogenes*, *Campylobacter jejuni*, *S. Enteritidis*, *E. coli*, and *S. aureus* (Beuchat 2000; Cressy *et al.* 2003).

Dorman and Deans (2000) reported on the antibacterial activity of 21 plant volatile oil components (including eugenol and linalool) against 25 bacterial strains by the agar well diffusion technique. Eugenol exhibited the widest spectrum of activity against 24 out of 25 bacteria, except for *Leuconostoc cremoris*, followed by linalool (against 23 strains, except *L. cremoris* and *Pseudomonas aeruginosa*). The MOBIC of clove oil was 50 μL mL^{-1}. At a very low concentration of 0.39 μL mL^{-1}, clove oil exhibited a very powerful radical scavenging activity similar to that of synthetic antioxidants (e.g., BHA, BHT) (Phoopuritham 2007; Phoopuritham *et al.* 2012). Shan *et al.* (2005) reported phenolic compounds to be the principal active components in spice extracts responsible for their AO capacity. The AO activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Rice-Evans *et al.* 1995).

CHAPTER 5

Efficiency of Antimicrobial and Antioxidant Food Packaging Systems: Role of Bioactive Compounds

SUNIL MANGALASSARY

5.1. INTRODUCTION

BIOACTIVE packaging is emerging as the main mode for moving towards sustainable food packaging. Antimicrobial and antioxidant packaging systems incorporating various bioactive compounds are an integral part of the bioactive packaging technology. These two systems are effective post-processing treatments in enhancing the quality and safety of various food products through reducing food pathogen proliferation and food spoilage. Universally, consumers are increasingly demanding minimally processed foods with more natural ingredients and preservatives. Therefore, bioactive antimicrobial and antioxidant agents play a crucial role in the future food safety and quality. According to Lopez-Rubio *et al.* (2006), bioactive packaging is a way to create healthier packaged foods which have a direct beneficial impact on consumer's health.

Bioactive compounds are compounds with a biological activity with an effect on living organisms. They are defined as essential and nonessential compounds that occur in nature and are part of the food chain with some health benefits (Biesalski *et al.* 2009). At present, both food and pharmaceutical industries have interest to obtain and characterize new bioactive compounds from natural sources (Chankvetadze and

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Cifuentes 2010). The types of bioactive compounds that have been proposed or used in food packaging include enzymes, peptides, polysaccharides, phospholipid analogs, antibodies, oligonucleotides, and other antimicrobial agents (Goddard and Hotchkiss 2007). Fruits and vegetables contain phytochemicals with antimicrobial and antioxidant properties along with many other biological activities such as antimutagenic, anticarcinogenic, and anti-inflammatory effects. Many essential oils possess antibacterial and antioxidant properties (Sahasavari *et al.* 2008).

5.2. ANTIMICROBIAL PACKAGING SYSTEMS

The basic function of food packaging is to extend the shelf life of food products through protection against chemical, physical, and biological contaminants. The role of food packaging has become more "functional" in recent years and the development of antimicrobial packaging systems fits well into that objective. Antimicrobial packaging is one of the major innovations within the broader realm of active packaging. Antimicrobial packaging can be defined in most simple terms as the incorporation of antimicrobial compounds into the packaging polymer matrix through different modes. It is intended to reduce, inhibit, or retard the growth of microorganisms that may be present in the packaged food. More recently, the development of antimicrobial packaging systems incorporating bioactive antimicrobial compounds in biodegradable packaging materials is a major step towards sustainable food packaging.

Antimicrobial packaging has been one of the most researched areas in food packaging for the last 10 years. Food science and packaging divisions of most of the universities around the world have been studying various aspects of this technology quite extensively. In spite of the above mentioned attempts, antimicrobial packaging has not yet been commercialized extensively in any of the developed countries. There are various factors that need to be given focus such as the effectiveness of this packaging system when manufactured in large scale, the ability of the antimicrobial agents to withstand some of the extreme conditions of packaging polymer manufacturing, and maintaining the antibacterial effect under various conditions of storage and transportation. Therefore, more attention should be given to develop this technology as a commercially feasible one with high efficiency.

5.2.1. Bioactive Compounds used Antimicrobial Packaging

Various classes of bioactive compounds that have been studied in antimicrobial packaging systems include bacteriocins, spices, and essential oils, enzymes, polysaccharides, and other bioactive phytochemicals (Coma 2008; Wang *et al.* 2012). Hauser *et al.* (2014) developed a solvent-based lacquer coating for food packaging films incorporating Maillard reaction end products as the antimicrobial agent.

5.2.2. Efficiency of Different Antimicrobial Packaging Systems

The efficiency of antimicrobial packaging systems depends on several factors such as their ability to inhibit microbial growth, sustain antimicrobial activity, and maintain the other essential quality characteristics of a packaging polymer including tensile strength and desirable barrier properties. In one of the earlier reviews of antimicrobial packaging concept, Appendini and Hotchkiss (2002) pointed out that the main rationale for incorporating antimicrobials into the packaging is to prevent surface microbial growth in foods where a large portion of spoilage and contamination occurs by achieving a gradual release of the antimicrobial compounds. The authors also stated that in order to exhibit the inhibitory effect, the packaging material must be in contact with the food product if nonvolatile antimicrobial compounds are used and therefore the surface characteristics and diffusion kinetics become significant. Several of the early research in the development of antimicrobial packaging has also demonstrated that antimicrobial release from polymer matrix has to be maintained at a minimum rate so that its surface concentration is above a critical inhibitory concentration (Vojdani and Torres 1989, 1990; Han and Floros 1998a, 1998b). Han (2000) identified the chemical structure of the polymer, production conditions, storage temperature, mass transfer coefficients, and physical properties of the polymer as some of the most important factors affecting the antimicrobial activity of the compound(s) incorporated.

Commonly, antimicrobial packaging systems are developed either by incorporation and immobilization of antimicrobial agents into the polymer matrix or by surface modification or surface coating. Another type of antimicrobial packaging is the use of polymers such as chitosan which possesses inherent antimicrobial activity. Another new approach is the use of antimicrobial nanostructures in the packaging materials.

5.2.2.1. Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins by lactic acid bacteria (Jack *et al.* 1995). The bacteriocins produced by Lactic acid bacteria are generally recognized as safe, have very little influence on gut microflora as they become inactivated by digestive proteases, they are pH and heat tolerant (Galvez *et al.* 2007). Commercially produced bacteriocins are nisin marketed as Nisaplin™ and pediocin PA-1 marketed as ALTA™ 2431 (Deegan *et al.* 2006).

Nisin, a bacteriocin produced by *Lactococcuslactis* subsp. *Lactis*, is probably the most researched and used bacteriocin in food safety applications including antimicrobial packaging. The primary site of action against vegetative cells is the cytoplasmic membrane, with nisin acting as a membrane depolarizing agent in a voltage dependent fashion. It acts on the cytoplasmic membrane forming transient pores which are dependent upon proton-motive forces and membrane lipid components. (Driessen *et al.* 1995; Delves-Broughton *et al.* 1996). Several studies of the development of antimicrobial packaging systems incorporating nisin have shown great efficiency. Nisin was approved for use in food in 1969 and was awarded generally recognized as safe (GRAS) status in the United States in 1988 (FDA 1988). Nisin is effective in many different types of food systems, inhibitory to many Grampositive food pathogens including *Listeria monocytogenes*. Nisin was also demonstrated to be effective against spores especially that of thermophilic bacteria such as *Bacillus stearothermophilus* (Delves-Broughton *et al.* 1996).

Nisin containing antimicrobial packaging systems have been tested for their efficiency using various methods. Dawson *et al.* (2003) evaluated the adsorption and release of nisin activity onto and from food grade powders to understand the mechanisms as a preliminary step to study the same mechanism in case of a polymer surface. They used different forms of silica and corn starch powders and tested for adsorption by placing the powders in agitated nisin solutions followed by the dehydration of the powder pellet after centrifugation. The dehydrated powders were then tested for inhibitory activity against either *Lactobacillus plantarum* or *Listeria monocytogenes*. The study showed that the nisin-adsorbed powders were highly efficient at both adsorption and release of antimicrobial activity. Immobilization of bacteriocins nisin (nisaplin) and lacticin 3174 to packaging materials was investigated by Scannel *et al.* (2000). Only nisin showed efficient adsorption onto the packaging material and its activity was retained for 3 months both under refrig-

eration and room temperature storage when tested against *Lactococcus lactis* subsp. *lactis* using modified agar diffusion assay. These authors also tested the antimicrobial packaging materials (polyethylene/polyamide pouches) to which nisin was immobilized at a concentration of 7860 AU/cm^2) against nonstarter lactic acid bacteria (NSLAB) *Listeria innocua* and *Staphylococcus aureus*. *L. innocua* populations were reduced by 2 logs by the second week by the nisin-adsorbed packaging material while the *S. aureus* populations showed reduction of 1 log after 12 weeks of storage. A study by Mauriello *et al.* (2005) tested nisin coated low density polyethylene (LDPE) film against *Micrococcus luteus* ATCC 10240 in tryptone soy broth (TSB) and found a significant reduction in count at 25°C.

Nisin containing antimicrobial films were also tested and found effective in reducing the bacterial contamination (both pathogenic and spoilage) of various food products. The antimicrobial activity of adsorbed nisin to cellophane surface was determined in fresh veal meat for effectiveness in reducing the total aerobic bacteria by Guerra *et al.* (2006).They found that the film resulted in approximately 1.5 log reductions in total aerobic counts through 12 days of storage at 4°C. In another interesting study, the researchers investigated the effect of antimicrobial sodium caseinate-based films containing nisin on surface and in-depth growth of *L. innocua* in cheese (Cao-Hoang *et al.* 2010). The films resulted in 1.1 log CFU/g reduction in *L. innocua* counts on the surface after 1 week of storage. But in in-depth inoculated samples, the antimicrobial effect was found to be dependent on the distance from the film contact surface to the cheese matrix. Many researchers had reported the loss nisin bioactivity when it comes in contact with the food (Aasen *et al.* 2003; Carnet Ripoche *et al.* 2006; Chollet *et al.* 2008) especially with fat and protein. Researchers were able to use an antimicrobial packaging film containing nisin, HCl, and EDTA to control spoilage microbiota in beef (Ercolini *et al.* 2010). The antimicrobial bags retarded the growth of LAB, carnobacteria, and *B. thermosphacta* for at least 10 days. The antimicrobial packaging also reduced the loads of enterobacteria from 1 to almost 3 logs compared to the control. This was explained by the use of EDTA in the nisin solution developed in this study. The EDTA can alter the outer membrane of the cell by chelating the magnesium ions that stabilize the membrane (Hancock 1984). In another study, cold-smoked salmon samples were surface inoculated with *L. monocytogenes* and then vacuum packaged with nisin-coated LDPE film and stored at 4°C or 10°C (Neetoo *et al.* 2008). The results

from this study showed that the nisin-coated films resulted in the reduction of bacteria at both temperatures, depending on the concentration of the nisin and initial inoculum level. A bacterially produced cellulose film containing nisin was developed and used to control *L. monocytogenes* and total aerobic bacteria on the surface of vacuum packaged frankfurters by Nguyen *et al.* (2008). Two concentrations of nisin (625 IU/ml and 2500 IU/ml) were used in this study and the results showed that only films containing higher level of nisin resulted in a significant reduction of *L. monocytogenes* after 14 days of refrigerated storage whereas both low and high concentrations of nisin had a significant reduction in total aerobic bacteria.

Nisin incorporated into packaging films along with other bioactive compounds also resulted in effective antimicrobial properties, i.e., Sivarooban *et al.* (2008) found that soy protein edible films containing grape seed extract, nisin, and EDTA effectively reduced major foodborne pathogens, *L. monocytogenes*, E. *coli* O 157: H7, and *Salmonella Typhimurium*. The inhibitory effect was more pronounced when the three compounds were used together than when used individually.

In addition to controlling food pathogens and total microbial load on food products, nisin containing antimicrobial films were tested to control biofilm formation on various food contact surfaces. A study carried out by Nostro *et al.* (2010) evaluated the effect of EVA films incorporating different concentration of nisin (0.1%, 0.5%, and 1%) on the biofilm forming ability *L. monocytogens*, *S. aureus*, and *S. epidermis*. Fluorescence microscopy confirmed poor biofilm formation on EVA-nisin film with the most pronounced effect shown on *S. epidermis* biofilms. Adding chelators like EDTA make nisin effective against Gram-negative bacteria also. Nisin's heat stability makes it a strong candidate to withstand high temperatures used in polymer extrusions.

Apart from nisin, other bacteriocins are also studied for their effectiveness in antimicrobial packaging systems. These compounds include pediocins, Enterocin 416K1, Lacticin 3147, and bacteriocin produced by *Lactobacillus curvatus* 32Y. In a study conducted by Iseppi *et al.* (2008), Enterocin 416K1, a bacteriocin produced by *Enterococcus casseliflavus* IM 416K1, was entrapped in a coating applied to LDPE film and evaluated against *Listeria monocytogenes*. In all the three methods of testing used (modified agar diffusion assay, quantitative determination in saline solution, and food testing), the coated film showed inhibitory effect on the organism. Pediocins are bacteriocins produced for some species of *Pediococcus* genera that exhibit a bactericidal effect

against some pathogenic Gram-positive bacteria (Cotter *et al.* 2005). Antimicrobial cellulose acetate films incorporating pediocin (25% and 50%) were tested for their inhibitory efficiency against *L. innocu*a and *Salmonella* sp. inoculated in ham (Santiago-Silva *et al.* 2009). The films were more effective against *L. innocua* (2 log reduction) during a 15 day storage compared to *Salmonella* spp. (0.5 log reduction). Mauriello *et al.* (2004) tested polythene films coated with a bacteriocin produced by *Lacotbacillus curvatus* 32Y in pork and ground beef inoculated with *L. monocytogenes* V7 and found that storage for 24 hours at 4°C resulted in a 1 log reduction of the organism.

5.2.2.2. Enzymes

A number of enzymes serve in nature to protect a biological system against invasion of certain microorganisms. Typical examples are lysozyme in egg albumen and lactoperoxidase in milk (Holzapfel *et al.* 1995). Lysozyme has been extensively studied as a food preservative in various systems including antimicrobial packaging systems (Corradini *et al.* 2013; Mecitoglu *et al.* 2006; Barbiroli *et al.* 2012). Lysozyme is mainly effective on Gram-positive bacteria by hydrolyzing β 1-4 glycosidic linkages between the N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer in the bacterial cell wall (Masuda *et al.* 2001). Similar to nisin, lysozyme has been also found to be effective against Gram-negative bacteria only when used in the presence of chelators such as EDTA and lactoferrin, which destabilizes the protective lipopolysaccharide (LPS) layer of Gram-negative bacteria, thereby giving access to the peptidoglycan layer (Ünalan *et al.* 2011). Mecitoglu *et al.* (2006) studied the antimicrobial effect of partially purified, hen egg white lysozyme incorporated alone and in combination with EDTA into zein film against *Bacillus subtilis*, *E. coli*, and *Lactobacillus plantarum*. The authors found that the combination of the two agents resulted in fully formed inhibition. The effect was more pronounced in the case of *B. subtilis* and least in *L. plantarum*. Also, lysozyme which is normally effective against Gram-positive bacteria was able to inhibit *E. coli* in the presence of the chelator, EDTA.

A few studies focused on the release mechanism of lysozyme from the polymer matrix and its effect on the resulting bacterial inhibition. The antimicrobial activity of lysozyme released from a monolayer cross-linked PVOH film and a multilayer structure made of cross-linked PVOH layers was studied by Buonocore *et al.* (2005). It was reported

that the released lysozyme caused cellular lysis of *Micrococcus lysodeikticus*. At the same time, the authors also noted that the effectiveness of the various films used in the study varied slightly depending on the structure of the film and the agents used in cross-linking. In another study, Gemili *et al.* (2009) achieved controlled release of lysozyme by changing the structure of a cellulose acetate film from highly asymmetric and porous to a dense one by modulating the composition of the initial casting solution. The results showed that increasing the cellulose acetate concentration resulting in a dense structure in turn caused a sharp decrease in the release rate due to higher mass transfer resistance in the matrix. The authors obtained maximum inhibitory effect against *E. coli* when they used 5% cellulose acetate and a combination of lysozyme and Na_2EDTA in the film forming solution. Fabra *et al.* (2014) investigated the effectiveness of pea protein and corn starch film containing lysozyme (0, 50, 75, 100 mg of lysozyme/g hydrocolloid) against *L. monocytogenes* and reported that the pea protein film containing a higher amount of lysozyme effectively inhibited the organism compared to the corn starch film. The results from the above mentioned studies clearly show that the antimicrobial effectiveness of the lysozyme depends on various factors such as the structure of the film, the release rate of the lysozyme (where a controlled rate is found more beneficial than a rapid release rate for sustained activity), the amount of the lysozyme, and the presence of chelating compounds such as EDTA.

5.2.2.3. Plant Based Bioactive Compounds

Plants synthesize aromatic substances, most of which are secondary metabolites and phenols or their oxygen substituted derivatives. The major groups of plant antimicrobials include phenolics and polyphenols, quinones, terpenoids and essential oils, lectins and polypeptides (Cowan 1999). Many of the plant derived bioactive compounds that have been tested in antimicrobial packaging system include different types of essential oils and their major components such as cinnamaldehyde, eugenol, thymol, linalool, and carvacrol. Other plant derived compounds such as allylisothiocyanate and gallic acid had also been incorporated and tested as antimicrobial packaging materials.

Essential oils are aromatic oily liquids obtained from various plant materials which contain a mixture of compounds such as terpenes, alcohols, acetones, phenols, acids, aldehydes, and esters (Corbo *et al.* 2009). Essential oils are secondary metabolites and play an important

role in plant defense and therefore most of them possess antimicrobial properties (Tajkarimi *et al.* 2010). Even though most of the essential oils show significant antimicrobial activity *in vitro*, their use as a food preservative is limited because high concentrations are needed to achieve sufficient antimicrobial activity (Hyldgaard *et al.* 2012). One of the important strategies to counteract the negative organoleptic effects of essential oils in foods mainly due to their intense aroma is to use them in active packaging rather than as an ingredient in the product itself (Hyldgaard *et al.* 2012). Essential oils can be encapsulated in polymers of edible and biodegradable coatings or sachets that provide slow release to the food surface or to the package headspace (Sánchez-González *et al.* 2011a).

Antimicrobial films prepared by incorporating different concentrations of bergamot, lemon, and tea tree essential oils, incorporated into chitosan and hydroxypropylmethylcellulose films were evaluated for their effectiveness against *L. monocytogenes*, *E.coli*, and *S. aureus* by Sánchez-González *et al.* (2011b). They reported that all three compounds were effective in inhibiting or reducing the three pathogens used. The antimicrobial activity of essential oils varied depending on the type of bacteria, the nature of essential oils, and the characteristics of the film matrix. All the three compounds showed higher efficiency against *L. monocytogenes* and *E.coli*. Cinnamaldehyde and allylisothiocyanate were incorporated into polycaprolactone films through solvent casting by Martinez-Abad *et al.*, (2013) to study the effectiveness against *S. enterica* and *L. monocytogens* using minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC), macrodilution, and vapor diffusion techniques. The films showed satisfactory inhibitory properties against the tested organisms. Interestingly, the authors tested the efficacy of the essential oils in the vapor phase on both organisms and found significant inhibition of both (94% and 55% inhibition for *Salmonella* and *Listeria*, respectively). Essential oils in the head space are more effective antimicrobials than their liquid phases counterparts, because lipophilic molecules in the aqueous phase associate to form micelles and prevent the attachment of the compound to the bacterial cells (Inoyue *et al.* 2003). Emiroğlu *et al.* (2010) studied the antimicrobial activity of soy edible films incorporating thyme and oregano essential oils against *E. coli*, *E. coli* O157:H7, *S. aureus*, *P. aeruginosa*, and *L. plantarum* by the inhibition zone test and also by inoculation challenge studies in ground beef. Among the bacteria tested, *L. plantarum*, and *P. aeruginosa* were the most resistant bacteria against oregano and

thyme essential oils in the inhibition zone tests. Beef coated with the prepared antimicrobial films showed varying degrees of antimicrobial activity with no effect on *Lactobacillus* and *Staphylococcus*, but a significant reduction in *P. aeruginosa*. A comprehensive study on zein films incorporating thymol for their effectiveness against three spoilage microorganisms (*Bacillus cereus*, *Candida lusitaniae*, and *Pseudomonas* spp.) and one GRAS microbial strain (*Streptococcus thermophilus*) was carried out by Del Nobile *et al.* (2008) using mathematical modelling, by fitting the Gompertz equation to the experimental data. The researchers found that films containing higher concentrations of thymol (20% and 30%) were most effective in controlling the growth of all the spoilage microorganisms tested including *B. cereus* spores. At the same time, the thymol films, at any of the tested concentrations, could not affect the viability of *S. thermophilus*. Another part of this study compared the antimicrobial efficiency of thymol incorporated films to thymol directly added to the inoculated growth medium in reducing the *Pseudomonas* spp. and found that differences were not significant. A study by Smith-Palmer *et al.* (2001) found that Gram-positive bacteria were more sensitive to essential oils than Gram-negative bacteria and reasoned the effect on the fact that the impermeable outer membrane of Gram-negative bacteria creates an obstacle for the compounds to penetrate the cell wall.

Polymer films (EVA) containing citronellol, eugenol, and linalool were developed and evaluated for their efficiency in controlling monospeciesbiofilm formation of *L. monocytogenes*, *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosaanddual* species (*S. aureus* and *E.coli*) for an incubation time up to 240 hours (Nostro *et al.* 2013). The results showed that antimicrobial activity increased with increasing oil concentrations and varied with the microorganisms used. The principal constituents of basil, linalool, and methylchavicol are GRAS substances and exhibit an antimicrobial effect against a wide range of microorganisms (Suppakul *et al.* 2003).The same authors (2008) investigated the feasibility of LDPE films containing linalool and methylchavicol to retard the microbial growth on food surfaces and found significant antimicrobial activity against *E. coli* in the agar diffusion disc test. The study also found that the films were effective in controlling the growth of *E.coli* and *L. innocua* in cheddar cheese during refrigerated growth.

5.2.2.4. Chitosan

Chitosan, a linear β-1,4-D-glucosamine, is a biocompatible, nontoxic

compound mainly obtained by deacetylation of chitin, a natural component present mainly in the exoskeleton of crustaceans (Fernandez-Saiz *et al.* 2008). Antimicrobial activity of chitosan has been demonstrated against many bacteria, filamentous fungi, and yeasts (Kong *et al.* 2010). Rabea *et al.* (2003) summarized the main bacteriostatic and bactericidal effects of chitosan to the binding of its positively charged amino groups to negatively charged carboxyl groups located on the surface of the bacterial cell membrane leading to disruption and leakage of the cell contents.

Antilisterial activity of chitosan-hydroxy propyl methyl cellulose (HPMC) films was determined on solid medium by Möller *et al.* (2004) and found to have significant inhibition of the organism. Ye *et al.* (2008) evaluated the antilisterial activity of chitosan coated plastic film alone or after incorporating five GRAS compounds. Chitosan film alone exhibited antimicrobial effect in culture medium in a concentration dependent manner, but could not control listerial growth in ham. Incorporation of antimicrobials into chitosan-coated films slowed down the listerial growth with sodium lactate showing maximum efficiency. Antimicrobial activity of chitosan films containing different concentrations of cinnamon essential oil was evaluated using agar diffusion method by Ojah *et al.* (2010). Incorporation of the essential oil at a level higher than 0.4% (v/v) resulted in clear zone when *L. plantarum* was used as the test organism. In this study, as also reported by Ye *et al.* (2008), chitosan film alone could not produce any significant inhibitory effect. Coma *et al.* (2002) postulated that chitosan does not diffuse through the agar media, but organisms in direct contact with the active sites of chitosan are inhibited. Bonilla *et al.* (2013) studied the antimicrobial properties of polylactic acid films containing different amounts of chitosan. All tested films containing chitosan resulted in significant reduction in total aerobic and coliform counts in pork meat stored for 7 days.

5.2.2.5. Combination with Other Preservation Techniques

Antimicrobial packaging systems can be efficiently combined with other preservation techniques to obtain a synergistic or additive effect. La Storia *et al.* (2012) used a polyethylene film coated with nisin for the packaging of beef steaks and stored under modified atmosphere conditions. There was a significant reduction in the total viable counts and some of the spoilage microflora using modified atmosphere packaging alone while a further reduction was obtained with the use of antimicrobial films. The authors concluded that the results of the study indicated

towards the fact that the combination of modified atmospheres and antimicrobial packaging assured an effect against Gramnegative and Grampositive spoilage associated populations. McCormick *et al.* (2005) studied the inhibitory effect of in-package pasteurization combined with a nisin containing wheat gluten film against *L. monocytogenes* and *S. Typhimurium* inoculated on refrigerated bologna during an 8-week storage period. The antimicrobial wheat gluten alone was effective in reducing the *L. monocytogenes* population, but not *S. Typhimurium*. Combining both treatments significantly reduced the *L. monocytogenes* population and prevented outgrowth during a 2 month storage period, but there was no added effect on *S. Typhimurium* compared to pasteurization alone.

5.2.2.6. Bio-Nanocomposites

A composite material results from the physical combination of two or more chemically distinct phases (a matrix and a dispersed phase) on a microscopic scale, separated by an interface. In nanocomposites, the dispersed phase is nanostructured (de Azeredo 2013). Nanocomoposite antimicrobial systems are found to be effective because of the high surface-to-volume ratio and enhanced surface reactivity of the nano-sized antimicrobial agents, enabling them to inactivate microorganisms more effectively than their micro- or macro-scale counterparts (Damm *et al.* 2008). Bioactive compounds such as chitosan, nisin, thymol, carvacrol, isothiocyanate, and lysozyme have been used in nanocomposite antimicrobial systems. An antimicrobial nanocomposite film is particularly desirable due to its acceptable structural integrity and barrier properties imparted by the nanocomposite matrix, and the antimicrobial properties contributed by the natural antimicrobial agents impregnated within (Rhim and Ng 2007). The use of mineral clays as antimicrobial carriers has been researched extensively. Nanoclays have a stacked arrangement of silicate layers with nanometric thickness. Montmorillonites (MMT) are layered silicate belonging to the structural family of the 2:1 phyllosilicates (de Azeredo 2013). Hong and Rim (2008) reported antibacterial activity from two organically modified montmorillonites, Cloisite 30B and Cloisite 20A.

5.3. ANTIOXIDANT FOOD PACKAGING SYSTEMS

Oxidation of fats is one of the leading causes of food spoilage apart

CHAPTER 10

Antimicrobial Edible Films

ARZU CAGRI MEHMETOGLU

10.1. INTRODUCTION

EDIBLE coatings prepared from polysaccharides, proteins, and/or lipids can be applied as thin coatings on the surface of foods. Interest in the development of antimicrobial edible films and coatings has continued to increase in responses to the ongoing concerns associated with both food spoilage and foodborne illnesses. Several studies have shown that antimicrobial edible coatings can be used to increase product shelf life and safety by inhibiting microbial growth (Cagri *et al.* 2004). Moreover, edible films or coatings applied on fruits and vegetables can extend their shelf-life by restricting water and oxygen transfer. When seasonings or antioxidants are incorporated, such films can also be used to deliver a wide range of flavors and delay lipid oxidation Another important reason for increased interest in edible films is the ongoing shift from nonbiodegradable (e.g., plastics) to biodegradable packing materials which includes edible films.

The concept of an edible film is by no means new with such films and coatings having been used for centuries. Initially, wax coatings were applied to citrus fruits in China as early as the 12th and 13th centuries (Hardenburg 1967). In the United States, melted paraffin wax coatings have been used commercially for oranges since the 1930s (Kaplan

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1986). These coatings were aimed to reduce moisture loss during transportation and storage after harvest. Later, coatings were used to improve product appearance, handling properties (M&M's® melt in your mouth), and shelf life (Kester and Fennema 1986). Currently, edible films and coatings are used in various applications, including casings for sausage and chocolate coatings for nuts and fruits. Edible films or coatings can also be developed to carry of a wide range of additives including antioxidants, vitamins, seasonings, preservatives, and antimicrobial agents. Application of such coatings on the surface of food products can reduce microbial growth, and thereby enhance both end-product safety and shelf life. In this chapter, antimicrobial edible coatings and their applications will be reviewed.

10.2. COMPONENTS OF EDIBLE FILMS

Edible films typically contain three major components: proteins, polysaccharides, and lipids. Proteins used in edible film include wheat gluten, collagen, corn zein, soy, casein, and whey proteins (Kester and Fennema 1986). Alginate, dextrin, pectin, chitosan, and cellulose derivatives are used in polysaccharide-based films (Kester and Fennema 1986). Suitable lipids for use in films include waxes, acylglycerols, and fatty acids (Debeaufort and Voilley, 1995; Park *et al.*, 1994). Composite films containing both lipid and hydrocolloid components also have been developed. Plasticizers are often added to film-forming solutions to enhance properties of the final film. These film additives are typically small molecules of low molecular weight and high boiling point that are highly compatible with the polymers (Banker *et al.* 1966). Common food-grade plasticizers such as sorbitol, glycerol, mannitol, sucrose, and polyethylene glycol decrease brittleness and increase flexibility of the film, which is important in packaging applications. Plasticizers used for protein-based edible films reduce protein interactions and increase both polymer chain mobility and intermolecular spacing (Lieberman and Gilbert 1973.). The type and concentration of plasticizer influences the properties of protein films (Cuq *et al.* 1997; Gueguen *et al.* 1998); mechanical strength, barrier properties, and elasticity decrease when high levels of plasticizer are used (Cherian *et al.* 1995; Galietta *et al.* 1998; Gondart *et al.* 1993). Water is another important plasticizer for protein films (Krochta 2002), but moisture content affects film properties. Common covalent cross-linking agents such as glutaraldehyde,

calcium chloride, tannic acid, and lactic acid are used to improve water resistance, cohesiveness, rigidity, mechanical strength, and barrier properties (Guilbert 1986; Marquis *et al.* 1995). Exposure to UV light will increase the cohesiveness of protein films by forming cross-links (Brault *et al.* 1997). Alternatively, enzymatic cross-linking treatments with transglutaminases or peroxidases can be used to stabilize films.

10.3. FILM-FORMING TECHNIQUES

Several techniques including solvent evaporation, thermal gelation, and solidification of melt have been used to produce edible films. Solvent removal is typically used to produce hydrocolloid edible films. In this process, a continuous structure is formed and stabilized by chemical and physical interactions between molecules. Macromolecules in the film-forming solution are dissolved in a solvent, such as water, ethanol, or acetic acid, that contains several additives (plasticizers, cross-linking agents, solutes). The film-forming solution is then cast in a thin layer, dried, and peeled from the surface. In preparing some types of protein films (whey protein, casein, soy protein, wheat gluten), the solution is heated for protein gelation and coagulation, which involves denaturation, gelation, or precipitation followed by rapid cooling. Intramolecular and intermolecular disulfide bonds in the protein complex are cleaved and reduced to sulfhydryl groups during protein denaturation (Okamota 1978). When the film- forming solution is cast, reformed disulfide bonds link the polypeptide chains together to produce the film structure, with the aid of hydrogen and hydrophobic bonding.

Melting followed by solidification is another common means for producing lipid-based films. Casting molten wax on dried methylcellulose films followed by solubilization of the methylcellulose can also be used to form wax films (Donhowe and Fennema 1993).

Extrusion is another technique to produce edible films in industry. Extrusion is a technique whereby a material placed into a cylinder, having a screw therein is mixed, kneaded, sheared, compressed, heated, and expanded by rotating the screw. With the extruding technique, it is possible to continuously perform two or more types of independent operations such as compressing, mixing, kneading, shearing, heating, and expanding simultaneously within a short time by placing the material in the cylinder installed of an extruder, rotating the screw, and extruding the material through a die. It is thus possible to design an effective

method for manufacturing edible films by using this technique, depending on the manufacturing conditions of the biodegradable molded articles and desired characteristics of final products.

10.3.1. Factors Affecting Film Properties

The specific applications for edible films are defined by a number of important film characteristics including water vapor permeability, oxygen permeability, tensile strength, elasticity, water or lipid solubility, and organoleptic acceptability. These properties are dictated by the various compenents in the film which may also include plasticisers, crosslinking agents, antimicrobial agents, antioxidants, and texture agents.

Edible films must generally be resistant to breakage and abrasion in order to strengthen the structure for food application. They also must be flexible in order to stretch around the product without breaking. The mechanical properties of edible films which depend on the structural forces between polymer molecules can be enhanced by the addition of plasticizers to the polymeric network that modify the energy between polymers by forming weak hydrogen bonds. Reducing the intermolecular forces between polymer chains will enhance the extensibility as well as the gas and water vapor permeability of edible films.

Permeability is defined as the extent to which water vapor or other gases can pass through the film matrix. Chemical composition plays a major role in the barrier properties of edible films. For example, polar polymers such as many proteins and polysaccharides show low gas permeability values but have poor moisture barrier properties. In contrast, nonpolar hydrocarbon-based materials such as lipids are excellent moisture barriers and less effective gas barriers. When added to polymer films, low molecular weight additives can improve or reduce the barrier properties of edible films depending on their chemical structure. Most edible film plasticizers increase water vapor permeability by disrupting polymer chain hydrogen bonding.

The characteristics of the permeant also influence its mobility through edible films, with smaller molecules generally diffusing faster than larger molecules, and polar molecules diffusing faster than nonpolar molecules, particularly in polar films. During permeation, adsorption of gases and water vapor on the film surface and the desorption through the opposite surface is also observed (Sperling 1992). These films also can have different barrier properties depending on their composition and method of production.

Addition of lipids to protein-based or polysaccharide-based edible films typically reduces their moisture permeability. The distribution of lipid particles within an emulsion-based film affects moisture permeability. Small lipid particles are more homogeneously distributed, which reduces water vapor permeability (Park *et al.* 1994a; Debeaufort and Voilley 1995; Perez-Gaco and Krochta 2001). However, during drying of the film-forming emulsion, solvent evaporation destabilizes the emulsion structure due to creaming, aggregation, and/or coalescence. The water vapor permeability of protein- and lipid-based films decreases as the drying temperature is increased, mainly because of changes in the emulsion structure (Perez-Gaco and Krochta 2001). Furthermore, the type of lipid type will impact water vapor permeability (WVP) of emulsified films. Generally, the water transmission rate of a film increases as the length of the lipid hydrocarbon chain decreases and the degree of unsaturation increases (Gennadios *et al.* 1993; Debeaufort *et al.* 1993; Park *et al.* 1994a). Hydrophobic alkanes and waxes, such as paraffin and beeswax, are the most effective barriers (McHugh and Krochta 1994c; Park *et al.* 1994a; Perez-Gaco and Krochta 2001).

Gelatinization and drying rates of hydrocolloidal film formula generally affect physical and mechanical properties of the film. For example, Flores *et al.* (2007a) showed that low gelatinization and drying rates increased tensile strength, elasticity, and degree of crystallinity of a film containing sorbate. In contrast, faster gelatinization and drying rates will decrease the mechanical and water vapor barrier properties due to the more amorphous structure of the film matrix.

10.3.2. Methods Used to Evaluate Antimicrobial Activity of Edible Films

Several studies have evaluated the effectiveness of antimicrobials in films and coatings. The method to be selected depends on the end-use of the film, the nature of the antimicrobial, and the characteristics of target microorganisms. In the film disk agar diffusion assay, a film disk containing the antimicrobial is placed on an inoculated agar plate and after incubation under specific conditions, the diameter of the zone where no growth occurred is measured. This test is generally applied as a screening step to test if the preservative is available to act as an antimicrobial in the film matrix (Cagri *et al.* 2001; Eswaranandam *et al.* 2004; Min *et al.* 2005a; Min *et al.* 2005b; Min *et al.* 2005c; Sanjurjo *et al.* 2006; Pintado *et al.* 2009). In this assay, diffusion of the antimicrobial from

the film disk depends on the size, shape, and polarity of the diffusing molecule, as well as the chemical structure of the film and pH and a_w of the agar (Cagri *et al.* 2003). This test is an end point assay and gives information on the ability of the antimicrobial incorporated in the film to inhibit microbial growth at a prefixed time.

Alternatively, microbial populations can be determined by plate count at selected times on the surface of inoculated agar plates in contact with antimicrobial film. This test is useful for assessing the efficacy of food wraps and gives information on whether the film has antimicrobial activity when in contact with an inoculated food surface (Coma *et al.* 2003; Kristo *et al.* 2008; Min *et al.* 2005a, Min *et al.* 2005b; Min *et al.* 2005c).

The film surface inoculation test is another frequently performed assay in which the target microorganism is inoculated on the surface of a film disk and then enumerated when the film is in contact with a semisolid medium such as agar that models a certain food product. This assay is used to simulate surface contamination. Results obtained may suggest what happens when microbial contamination occurs on coatings or films in contact with a food and gives an idea of the barrier capacity of the film to prevent external contamination (Flores *et al.* 2007b; Sanjurjo *et al.* 2006; Vásconez *et al.* 2009). The methods mentioned have been used for in vitro evaluation of antimicrobial film performance.

When the film or coating is applied to the food, antimicrobial effectiveness is evaluated by enumerating the indigenous microflora and/or inoculated target organism during storage (Martins *et al.* 2010; Mitrakas *et al.* 2008; Moreira *et al.* 2009; Seol *et al.* 2009). Efficacy of the antimicrobial agent is dictated by the rate at which it is released from the film. For some applications, quick release of the antimicrobial is required to control microbial growth in the food; whereas in other cases, a much slower release may be required to assure a certain level of preservative at the surface. The release rate and extent of antimicrobial activity required over time are important considerations when attempting to optimize antimicrobial activity for specific applications.

10.4. ANTIMICROBIAL EDIBLE FILMS

Various antimicrobial edible films have been developed to control the growth of spoilage and pathogenic microorganisms that may con-

taminate the surface of foods after processing. In most solid foods, contamination and microbial growth occur on the food surface, which leads to a reduction in product shelf life. Edible films containing various antimicrobials such as benzoic acid, sorbic acid, propionic acid, lactic acid, nisin, and lysozyme have been used to retard the growth of bacteria, yeasts, and molds on different product surfaces (Table 10.1). Antimicrobial edible films were broadly discussed in several review articles (Cagri *et al.* 2004; Dong *et al.* 2004; Cha *et al.* 2010; Valencia-Chamorro *et al.* 2011; Campos *et al.* 2011).

10.4.1. Diffusion of Antimicrobial Agents from Edible Film

The primary advantage of antimicrobial edible films is that the inhibitory agents in these films can be specifically targeted to contaminants on the food surface, with the diffusion rate of the antimicrobial into the product partially controlled by entrapment in the film matrix. In one study, lactic acid–treated casein films containing sorbic acid were tested on the surface of intermediate moisture papaya cubes inoculated with *Staphylococcus rouxii* or *Aspergillus niger* (Guilbert 1988). Casein films retained 30% of their original sorbic acid content after 30 days of storage at 95% relative humidity, with no growth of either test organism observed. However, complete diffusion of sorbic acid into the fruit was observed in the absence of the film in control samples after 24 hours of storage, confirming that the edible film matrix entrapped the antimicrobial and reduced diffusion during storage.

Controlling the antimicrobial release from edible films is very important. Release of antimicrobial substances from edible films is dependent on many factors, including electrostatic interactions between the antimicrobial agent and polymer chains, ionic osmosis, and structural changes induced by the presence of antimicrobial and environmental conditions.

Diffusivity of sorbic acid from edible films of different materials was evaluated by several researchers (Torres *et al.* 1985; Guilbert *et al.* 1985; Giannakopoulos and Guilbert 1986; Guilbert 1988: Vojdani and Torres, 1989). They showed that sorbic acid permeability could be affected by film composition. For example, the addition of palmitic acid reduced sorbic acid release by about 65% from methycellulose edible films and by 75% from hydroxymethylcellulose edible films, respectively (Vojdani and Torres 1989). The same research group also reported increasing lipid derivative concentrations with the presence of

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