CHAPTER 2

LITERATURE REVIEW

2.1 Biodegradable films

The use of plastic packaging by the food industry has increased at an accelerated rate due to cost considerations and their inherent properties. However, environmental awareness has stimulated a need for use of degradable and renewable resources. Research on alternative uses and reprocessing of biopolymers will add commercial value and reduce pollution problems (Garcia *et al*., 2004). Biodegradable films can then be used to replace some petroleum-based films. However, biodegradable films should meet a number of requirements, such as moisture and gas barrier, appearance, mechanical characteristics and non-toxicity.

2.1.1 Chitosan

Chitosan is derived from chitin, which is the second most abundant polysaccharide on earth. Chitin is obtained as a by-product from waste materials from the fishing industry. Chitosan is a high molecular weight polymer composed of N-acetyl-D-glucosamine units connected via ß (1–4) glycosidic bonds (Figure 1). Chitosan is inherently antimicrobial and has been used in films and coatings. Chitosan is positively charged (natural cationic polysaccharide) which promotes cell adhesion, and the free amino groups interfere with the negative charges of bacterial cell membranes, causing leakage of intracellular constituents (Goldberg *et al*., 1990). Furthermore, this positive charged nature provides the film with good mechanical and oxygen barrier properties (Chen *et al*., 1996; Caner *et al*., 1998).

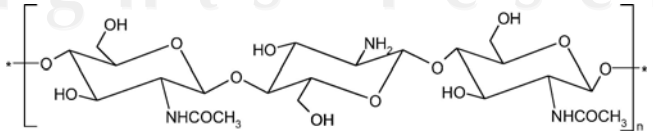

Figure 2.1 Structure of chitosan (Moller *et al*., 2004).

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Properties of chitosan film depend on the source of the chitosan (molecular weight and degree of deacetylation), solvent used, drying condition, storage time, temperature, etc. Park *et al*. (2002) reported that increasing molecular weight of the chitosan molecules resulted in improving strength of the films but did not significantly affect water vapor permeability (WVP). Muzzarelli and Peter (1997) discovered that higher molecular weight chitosan have good film-forming properties as a result of intra- and intermolecular hydrogen bonding. Cervera *et al*. (2004) found that high molecular weight chitosan absorbs more water than low molecular weight chitosan. Kim *et al*. (2006) studied the effect of the degree of deacetylation on properties of chitosan film. They found that low degree deacetylated chitosan films had lower WVP and higher tensile strength compared with highly deacetylated chitosan films. The elongation values were not affected by degree of deacetylation. As pH increased, WVP of chitosan film tended to increase while tensile strength decreased significantly. Highly deacetylated chitosan film was very sensitive to pH change in comparison to low degree deacetylated chitosan film. With increasing pH value, the degree of dissociation of chitosan decreased. High deacetylated chitosan has more amino groups that can be dissociated in an acid solvent than low deacetylated chitosan. Chitosan is not soluble in pure water or organic solvents but it is soluble in slightly acidic solutions. Caner *et al*. (1998) studied the effect of different acids; acetic, formic, lactic and propionic acids, on their ability to dissolve chitosan. They discovered that acetic acid is the best solvent to use to produce a film with high water barrier and good mechanical properties. Kim *et al*. (2006) also showed that the WVP of chitosan film was low for acetic and propionic acids and unaffected by pH (in a range of 3-5). The tensile strength of chitosan film dissolved in aqueous acetic acid was the highest. Srinivasa *et al*. (2004) demonstrated the effect of drying on chitosan film properties. Air drying produced a stronger and more transparent film than oven drying. However, oven drying produced a film having a higher barrier to oxygen and water and a greater amount of crystallinity. Kato *et al*. (1978) showed that elevated temperature enhanced the crystallinity due to hydrophobic interactions. Caner *et al*. (1998) found that the elongation of chitosan film decreased with storage time.

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The ability of films to modify gas transport is important for tailoring films to specific applications such as fresh fruit and vegetables (Guilbert *et al*., 1996). The gas permeability of films from biopolymers depends on the nature of the gas, the structure of the material, temperature and moisture conditions (Gontard *et al*., 1994). When there are no pores, faults, or membrane punctures, permeability (P) is equal to the product of the diffusion coefficient (D), representing the mobility of permeant molecules in the polymer and the solubility coefficient (S), representing the permeant concentration in the film balanced with the external pressure: P = DS. D is constant with permeant concentration and time, and the Henry's law of solubility was applied. Increasing in permeability can thus be related to increasing in the diffusion coefficient, due to structural modifications, and also to increase in oxygen solubility in the film due to an increasing water content in the film (oxygen solubility in water = 1.25 mmol/L at 25°C and 1 atm). Generally, carbon dioxide permeates through plastic polymer more rapidly than other atmospheric gases such as oxygen. The solubility of carbon dioxide in water (34.5 mmol/L at 25°C and 1 atm) is higher than for oxygen. Water molecules in the polymer interact with hydrophilic groups and may thus disrupt hydrogen bonding, creating additional sites for the dissolution of oxygen and increased mobility of oxygen molecules within the polymer. Films with high CO_2/O_2 ratio values will allow carbon dioxide to escape from the package relatively easily, resulting in low carbon dioxide concentration atmosphere. Since fruit and vegetables vary in their tolerance to carbon dioxide and in their ability to benefit from high carbon dioxide percentages. The selectivity ratio value (ratio of carbon dioxide permeability to oxygen permeability) of a film is very important for predicting the relative amounts of oxygen and carbon dioxide that will accumulate in a package headspace. At very high relative humidity (RH) close to 100%, solubility of oxygen or carbon dioxide molecules in the free water of the film becomes the main parameter for the transport of these molecules through the film. Microperforated synthetic films are marketed for their ability to achieve high permeability by allowing gases to move across the film via channeling, which is much faster than the usual permeation process. However, channeling does not provide the differential permeabilities to oxygen and carbon dioxide that nonperforated films offer with resulting CO_2/O_2 permeability ratio values close to 1. Since both product respiration and film permeability are

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temperature sensitive, it is essential to determine PO_2 and PCO_2 at the low temperature at which the film will be stored (Gontard *et al*., 1996).

2.1.2 Methylcellulose

Cellulose is the most abundant natural polymer on earth. Methylcellulose is a nonionic cellulose ether which has an excellent film-making ability. Methylcellulose makes strong, tough and flexible water-soluble films. It is transparent and oil resistant. Methylcellulose film has excellent barrier to oxygen and aroma compounds (Donhowe and Fennema, 1993a, b; Park *et al*., 1993; Nisperos-Carriedo, 1994). It is also selectively permeable to CO_2 and O_2, and hence, retards the respiration and ripening of many fruit and vegetables by limiting the availability of O_2. Hydrophilic films and coatings, such as polysaccharides, provide a good barrier to CO_2 and O_2 under certain conditions, but a poor barrier to water vapor (Guilbert 1986; Park and Chinnan 1995). Methylcellulose film is hydrophilic because it contains hydroxyl groups and it is also poor moisture barriers (Barrie, 1968; Kamper and Fennema, 1984; Pascat, 1985; Schwartzberg, 1985; Biquet and Labuza, 1988; De Leiris, 1994). The poor water vapor barrier property allows for the movement of water vapor across the film, thus preventing water condensation that can be a potential source of microbial spoilage in horticultural commodities (Park *et al*, 1994). Turhan and Sahbaz (2004) pointed out that the WVP of methylcellulose film was 25–500 times greater than those of synthetic films. However, these films are better water vapor barriers than hydrophilic films based on starch, casein, and wheat gluten (Allen *et al*., 1963; Greener and Fennema, 1989b; Kester and Fennema, 1989; Aydt *et al*., 1991; Gontard and Guilbert, 1994). To alleviate this weakness, methylcellulose has been combined with lipids to improve its barrier properties to water vapor, oxygen and carbon dioxide (Kamper and Fennema, 1984; Greener and Fennema, 1989a,b; Kester and Fennema, 1989; Rico-Pena and Torres, 1990; Koelsch and Labuza, 1992). Ayranci *et al*. (1997) discovered that the WVP of methylcellulose films was found to decrease with increasing molecular weight above a molecular weight of 41,000 daltons. In general, the decrease in permeability with increasing molecular weight of the cellulose component may be explained by the possibility that the mobility of the molecule decreases with increasing molecular weight. Thus its contribution to water vapor

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transfer becomes less. On the other hand, Park *et al*. (1993) found that WVP values of edible films increased with increasing molecular weight of methylcellulose. An important component of edible films is the plasticizer. Polyethylene glycol (PEG) is one of the most widely used as edible film plasticizer. WVP values were found to decrease sharply with increasing molecular weight of PEG up to about 1000, whereas a slight increase was observed above this level. Transparency of films was observed to decrease as the molecular weight of PEG was increased. Turhan *et al* (2001) explained that in methylcellulose the proton donor is the hydroxyl group and the proton acceptor is the oxygen. Therefore, hydrogen bonding between methylcellulose molecules affects the stiffness, hardness and brittleness of the film. PEG400 is a relatively small hydrophilic molecule and can be easily inserted between methylcellulose chains. Incorporation of PEG into the polymer matrix decreased the attractive forces between methylcellulose chains, increased free volume and segmental motions, improved flexibility and extensibility hence water molecules diffused more easily. The result from Fourier transform infrared spectroscopy (FTIR) showed the formation of hydrogen bonding between methylcellulose and PEG, thus reducing intermolecular attraction between methylcellulose chains. Hydrogen bonding interactions decreased as the molecular weight of PEG increased. PEG400 has a larger number of hydroxyl groups per mole compared to higher molecular weight PEG and its small size facilitates the penetration of this plasticizer into the polymer matrix. The addition of plasticizers increased the hydrophilicity of films by exposing their hydroxyl groups. In addition, as the molecular weight of PEG increases, its polarity and solubility decrease, reducing its ability to interact with polymer chains.

Debeaufort and Voilley (1997) did not observe a glass transition on pure methylcellulose films, but did observe a glass transition on methylcellulose-PEG400 films. They concluded that PEG400 does not have much interaction with the methylcellulose matrix. The plasticizing effect is probably due to a lubrication phenomenon between the polymer chains because PEG400 has no or a very low compatibility with the methylcellulose matrix. These conclusions are not in agreement with the work of Donhowe and Fennema (1993b), who found that PEG400 is compatible with methylcellulose up to a concentration of 30%. Debeaufort and

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Voilley (1997) also found that water has a greater effect on tensile strength than on elongation whatever the concentration of PEG400. On the contrary, PEG400 acts more as a lubricant of the polymer chains than as a plasticizer. Thereby, it allows the methylcellulose fibers to slide one over the other, thus increasing the film elongation.

Turhan and Sahbaz (2004) studied the solubility of methylcellulose film. They found that methylcellulose film was water-soluble and films containing PEG had higher solubility. The high dissolution rate indicated the low cohesion of methylcellulose via numerous hydrogen bond between methylcellulose chains (Turhan *et al*., 2001). PEG disrupted of native three-dimensional structure of methylcellulose through hydrogen bond formation with PEG. Intermolecular hydrogen bond between methylcellulose chains were replaced by hydrogen bond with PEG. Methylcellulose also dissolved in an aqueous ethanol solution.

Turhan *et al*. (2001) demonstrated that neither ethanol nor its concentration modifies the structure of the polymer. Due to its low boiling point (78ºC), it reduces drying time and does not remain in the film after drying. Similar conclusions were expressed by Debeaufort and Voilley (1997), who found that ethanol did not change the structure and thermal properties of methylcellulose. Donhowe and Fennema (1993a) observed an increase in crystallinity in methylcellulose films at higher drying temperature.

2.1.3 Composite films

Composite films are generally designed to take advantages of the properties of the individual components. Generally, films composed of one primary substance either act as good barriers or have good mechanical properties, but not both. Polysaccharide and protein films have good barrier to oxygen but are hydrophilic. Lipid films provide good moisture barrier, but are weak. The mechanical and barrier properties of composite biopolymer films strongly depend on the constituting polymer characteristics and their compatibility (Donhowe and Fennema, 1993b; Butler *et al*., 1996).

Gracia *et al*. (2004) discovered that methylcellulose film was completely soluble in water at both 25^{o}C and 100^{o}C while chitosan films had low solubilities; below 4.5% at 25^{o}C and 11.5% at 100^{o}C after 1 hour dipping; as expected, solubility

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increased with temperature. Chitosan/methylcellulose films had WVP values similar to those of cellophane, as expected due to the similar chemical structure of the components. Ayranci and Tunc (2001) studied the effect of fatty acid types on properties of methylcellulose film. WVP decreased with increasing fatty acid content. This is mainly due to the hydrophobicity of fatty acids present in the film composition. They found that stearic acid, even at the lowest content level studied (5 g stearic acid/100 g methylcellulose), was the most effective in decreasing both the water vapor and the CO_2 transmissions of the films with addition of stearic acid. WVP of the film dropped by about 40%. This behavior can be explained by increasing hydrophobicity of the fatty acids with increasing chain length in the order of lauric acid (C12), palmitic acid (C16) and stearic acid (C18). Furthermore, the chain mobility of fatty acids, which helps in the transmission of water vapor, decreases in the same order. Chain mobility depended mostly on molecule size or length. The bigger molecules resulted in the bulky and impeded movement. Stearic acid possesses the longest hydrocarbon chain, and the most hydrophobic and has the lowest chain mobility. The incorporation of stearic acid into the composite chitosan/hydroxypropyl methylcellulose (HPMC) film formulation decreased initial solubility in water and increased water drop angle (Moller *et al*., 2004). Stearic acid content more than 15% (w/w HPMC) produced an optically unstable film with very poor mechanical properties (Coma *et al*., 2003) because of a discontinuous structure. The emulsion of fat in water based polymer was not well miscible. Debeaufort *et al.* (1995) observed that incorporation of solid fat into methylcellulose films did not affect their mechanical properties. Cross-linking agents also affected the permeation of water in films. Cross-linking of composite chitosan/HPMC with citric acid led to a 40% reduction in its solubility in water (Moller *et al*., 2004).

Wu *et al*. (2004) reported that the mechanical properties of the cellulose/chitosan blends appeared to be dominated by cellulose, which suggests that the cellulose/chitosan mixture was not well blended. Chitosan has poor tensile strength when wet. Blending cellulose with chitosan can be expected to improve the mechanical properties of chitosan. Several studies have reported that specific interactions occurred between cellulose and chitosan molecules based on the analysis of Raman and 13C NMR spectroscopy, and X-ray diffractometry (Hasegawa *et al*.,

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1992a,b; Isogai and Atalla, 1992; Hasegawa *et al*., 1994). Gracia *et al*. (2004) and Pinotti *et al*. (2007) found that chitosan film showed rigid characteristics (high elastic modulus and small elongation). Film flexibility increased with increasing methylcellulose content. The higher elongation and lower elastic modulus of composite films indicated the importance of hydrocolloid interactions. Xu *et al*. (2005) found that the tensile strength of the chitosan/starch composite films increased with the addition of starch and reached a maximum at a starch to chitosan ratio of 1:1. The increasing tensile strength was attributable to the formation of inter-molecular hydrogen bonds between the amino groups of the chitosan backbone and the hydroxyl groups of the starch. This was confirmed by FTIR. The tensile strength then decreased abruptly with further increase of the starch to chitosan because of phase separation between the two main components. Using X-ray diffraction, Xu *et al*. (2005) reported that the chitosan powder was in a crystalline state. After making the films, two crystalline peaks still existed, but were less intense. The crystalline structure still existed in the regular starch film, whereas an amorphous state was observed in the waxy starch film. When these two film-forming components were mixed at a starch to chitosan ratio of 0.5:1, two chitosan peaks were still present at this low ratio. However, the crystalline peaks of the chitosan were suppressed when the starch ratio in the composite film was increased.

Cervera *et al*. (2004) reported that there are clear changes in the crystallinity of the chitosan-amylose maize starch films during storage. The changes seem to be dependent on the storage conditions and the plasticizer used. Furthermore, the films became more flexible and less brittle when stored at a high relative humidity. More crystallinities were present in the films stored at 25^{o}C/60% RH than of those stored at 40^{o}C/75% RH. After 2 months, the diffraction pattern of the 40^{o}C/75% RH sample had a strong amorphous profile.

Color modification during storage is commonly reported for protein films due to Maillard reactions. Rancidity was found in lipid-based films (Trezza and Krochta, 2000b). Polysaccharide films are free of these problems. Cervera *et al*. (2004) reported that the yellow tint of the chitosan/amylose maize starch films became more evident as the films were stored at higher temperature and relative humidity (40.8^{o}C/75% RH).

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2.2 Antimicrobial biodegradable films

The growth of microorganisms on the surface of the products is a main cause of food spoilage. The mixing of antibacterial substances directly into a food has some limitations because the active substance can be neutralized, and evaporated off, or they may inadequately diffuse into the bulk of the food (Torres *et al*., 1985; Siragusa and Dickson, 1992). The incorporation of antimicrobial agents into packaging can create an environment inside the package that may delay or prevent the growth of microorganisms on the surface of the products and, hence, lead to an extension of its shelf life (Han, 2000). Antimicrobial packaging encompasses any packaging technique(s) used to control microbial growth in a food product including packaging materials containing antimicrobial agents. Antimicrobial packaging has attracted much attention from the food industry because of the increase in consumer demand for minimally processed, preservative-free products. Reflective of this demand, the preservative agents (preferably natural preservatives) must be applied at the lowest effective level (Cha and Chinnan, 2004). There are three basic categories of antimicrobial films (Cooksey, 2001):

1) Incorporation of the antimicrobial additive directly into the packaging film.

2) Coating the antimicrobial additive on a material that acts as a carrier.

3) Inherently antimicrobial polymer with film-forming properties such as cationic amino-polysaccharides.

Chitosan has been used as a coating and appears to protect fresh vegetables and fruit from fungal degradation. Chitosan has two functions; it has antifungal activity and acts as a barrier between the produce and microorganisms (Cuq *et al*., 1995). Chitosan has intrinsic antimicrobial activity (Papineau *et al*., 1991; Sudarshan *et al*., 1992; Wang 1992). Positively charged chitosan molecules interact with negatively charged bacteria membranes causing disruption and death of the cell (Young and Kauss, 1983; Helander *et al*., 2001). The antimicrobial activity of chitosan is active at below pH 6.0. Sudharsan *et al*. (1992) reported that chitosan was no longer bactericidal at pH 7.0 because of two major reasons: the presence of a significant proportion of uncharged amino groups and the poor solubility of chitosan in pH 7.0. Coma *et al*. (2003) showed that chitosan inhibited the growth of *Staphylococcus aureus* for at least 10 days on nutrient agar. Antimicrobial properties

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of chitosan films also depend on molecular weight and degree of acetylation. Hernandez-Lauzardo *et al*. (2008) reported that the low molecular weight chitosan was more effective for inhibition of mycelial growth while the high molecular weight chitosan affected spore shape, sporulation and germination. Wang (1992) observed that chitosan with a degree of acetylation of 7.5% was more effective than chitosan with a degree of acetylation of 15%. Chitosan (1-1.5%) is required for complete inactivation of *Staphylococcus aureus* after two days of incubation at pH 5.5 or 6.5 in the medium. Chitosan (0.5-1%) completely inactived *Escherichia coli* after a 2-day incubation at pH 5.5. He also reported that complete inactivation could be obtained even after the first day, if the chitosan concentration was more than 1% in the broth. Simpson *et al*. (1997) found that only 0.0075% chitosan was needed to inhibit the growth of *Escherichia coli*. Hernandez-Munoz *et al*. (2008) observed that no fungal decay on strawberry coated with 1.5% chitosan (with or without the addition of calcium gluconate). By contrast, 12.5% strawberries coated with 1.0% chitosan without calcium gluconate were infected after 5 days storage. Wu *et al*. (2004) reported that a chitosan/cellulose blend demonstrated effective antimicrobial capability against *Escherichia coli* and *Staphylococcus aureus*. Ponce *et al*. (2008) found that the use of chitosan coatings enriched with rosemary and olive oil oleoresins applied to butternut squash did not produce a significant antimicrobial effect. The reduction in total bacterial counts was observed in all sample treated with rosemary and olive oleoresins. No combinations of film-oleoresins conferred significant advantages regarding the antibacterial properties observed. A possible explanation could be that the antibacterial compounds would be dispersed in the coating and thus bacterial cells would be less exposed to them. Antimicrobial agents may gradually migrate from the film to the food surface, producing their antimicrobial effects during storage.

Furthermore, other compounds in the system (type and concentration of the acids, presence of proteins, lipids, ions, and other food ingredients), and environmental conditions (temperature and relative humidity) also affected antimicrobial activity and mechanical properties of chitosan (Begin and Van Calsteren, 1999; Kurita, 2001; Synowiecki and Al-Khateeb, 2003; Zheng and Zhu, 2003). Caner *et al*. (1998) and Begin and Van Calsteren (1999) reported that type of acid used for

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film preparation significantly affected film properties. Films prepared with acetic and formic acids had the highest tensile strengths followed by those found with lactic, propionic, and citric acids. Jiang and Li (2001) pointed out that the interaction between the preservative agents and the film-forming materials may affect release of the preservatives and the mechanical properties of the films**.** Moller *et al*. (2004) found a loss of antimicrobial activity in chitosan/HPMC blends after chemical cross-linking modification by citric acid. According to Brody *et al*. (2001), the antimicrobial effect of chitosan occurs when organisms are in direct contact with the active sites of chitosan. When antimicrobial agents are incorporated into a film, there will generally be some diffusion out of the film, thus improving its antimicrobial efficacy. The diffusion itself is dependent on the size, shape and polarity of the diffusing material. The chemical structure and the cross-linking level of the films also affect this phenomenon (Cagri *et al*., 2001).

2.3 Vanillin

Many consumers have concerns over the addition of chemical additives to food, this has driven the food industry and food research organizations search for natural antimicrobial compounds (Devlieghere *et al*., 2004b). Natural antimicrobials are GRAS (Generally Recognized as Safe) and thus herbs and spices are of interest. Phenolic compounds are the major active antimicrobial components present in some essential oils such as eugenol, carvacrol, thymol, and vanillin (Lopez-Malo *et al*., 2000). Phenolic antimicrobials are generally regarded as membrane active compounds due to their hydrophobicity (Davidson,1993; Juven *et al*.,1994; Helander *et al*.,1998; Ultee *et al*.,1999; Davidson and Naidu, 2000). Kabara (1991) mentioned that undissociated phenolic groups were more active on microorganisms than dissociated forms, suggesting that they can act at a wide pH range (3.5–8.0). Prindle and Wright (1977) stated that the effect of phenolic compounds was concentration dependent. At low concentration, phenols affected enzyme activity, especially of those enzymes associated with energy production, while at greater concentrations caused protein denaturation. Conner and Beuchat (1984a, b) suggested that the antimicrobial activity of essential oils on yeasts could be the result of disturbance in several of the enzymatic systems involved in energy production and structural

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components synthesis. Once the phenolic compounds crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity. The use of spices, herbs, plants, essential oils, and related phenolic compounds as antimicrobials is limited due to the high minimum inhibitory concentrations (MIC) required in foods with high protein and/or fat content (Lopez-Malo *et al*., 1995, 2000), which may impart objectionable flavors and/or aromas. Essential oils can have an adverse effect on the sensory characteristics of food products especially minimally processed fruit and vegetables.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major constituent of vanilla beans and is a principle flavor compound in numerous foods. Vanillin (Figure 2.2) has been reported to act as an antioxidant (Burri *et al*., 1989). Moreover, recent reports have shown that vanillin can be effective in inhibiting bacteria, yeasts and molds as seen in Table 2.1.

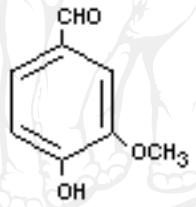

Figure 2.2 Structure of vanillin (Fitzgerald, *et al*., 2005).

The antimicrobial activity of vanillin depends on the time of exposure, concentration and the target organism. Fitzgerald (2004) observed that vanillin inhibited *Escherichia coli, Lactobacillus plantarum and Listeria innocua* and was found to be bacteriostatic. Jay and Rivers (1984) reported that the inhibitory activity of vanillin was more effective against non lactic Gram-positive than Gram-negative bacterias. However, Fitzgerald (2004) reported that *Escherichia coli* had the lowest minimum inhibitory concentration (MIC) compared with *Lactobacillus plantarum and Listeria innocua,* indicating that certain Gram-negative bacteria can be equally susceptible to the antimicrobial activity of vanillin. Moon *et al*. (2006) reported that 40 mM vanillin exerted a lethal effect on *Escherichia coli* 0157:H7. *Escherichia coli* was more sensitive to vanillin than *Listeria monocytogenes*, and viable cells could not

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be recovered after 2 days incubation at either 5°C or 14°C. *Listeria monocytogenes* and *Escherichia coli* 0157:H7 were inoculated at 10^5 colony forming units (CFU)/ml in pH adjusted (pH 4.00) or unadjusted (pH 3.42) juice from Granny Smith apples that were supplemented with 40 mM vanillin. Neither species were recoverable after 3 days incubation at both temperatures. Rupasinghe *et al*. (2006) reported that total aerobic counts of untreated fresh-cut apple slices increased by 4.3 log CFU/g fresh weight from day 1 to day 19 at 4°C. In comparison, the total microbial loads of NatureSeal (antibrowning agent) plus 12 mM vanillin treated apple slices was on average only 1.6 log CFU/g fresh weight. Cerrutti *et al*. (1997) treated strawberry puree with a mild heat treatment combined with 3,000 ppm vanillin and 500 ppm ascorbic acid. Water activity (a_w) was adjusted to 0.95 and pH to 3.0. They found that native and inoculated flora were inhibited for at least 60 days storage at room temperature. The authors concluded that vanillin provided the bactericidal effect. Penney *et al*. (2004) found that 2,000 ppm vanillin significantly suppressed fungal and total microbial growth in yoghurt over a 3 week period. They mentioned that the lack of lethality of 1,000 ppm vanillin in yoghurt may have been due to a higher level of fat and/or protein, which may have reduced its activity (McNeill and Schmidt, 1993).

Vanillin also provides an inhibitory effect against yeast and mold. Vanillin (13 mM) inhibited the growth of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii, Debaryomyces hansenii* and *Zygosaccharomyces rouxii* in culture medium and in apple puree for 40 days. However, vanillin was less effective in banana puree and 20 mM was insufficient to inhibit the growth of *Zygosaccharomyces bailii*. The authors concluded that the higher lipid/protein levels in bananas interfered with antimicrobial activity of vanillin (Cerrutti and Alzamora, 1996). Fitzgerald *et al*. (2003) reported that increasing levels of vanillin reduced growth yield and increased both the lag and the doubling times of the yeast cultures. Complete inhibition of cell growth was observed at a concentration of 20 mM vanillin. However, given the strong aromatic flavor of vanillin, only lower levels could be added if vanillin was to be used as a wide spread preservative, so as not to affect the organoleptic qualities of the food.

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Table 2.1 Inhibition effect of vanillin on a variety of microorganisms.

Microorganisms	MIC	Conditions	Reference
Escherichia coli	15 mM		Fitzgerald (2004)
Lactobacillus plantarum	75 mM		Fitzgerald (2004)
Listeria innocua	35 mM		Fitzgerald (2004)
Debaryomyces hansenii	2,000 ppm	apple puree, 40 days	Cerrutti and Alzamora (1996)
Saccharomyces cerevisiae	21 mM		Fitzgerald (2003)
Saccharomyces cerevisiae	2,000 ppm	apple puree, 40 days	Cerrutti and Alzamora (1996)
Zygosaccharomyces bailii	2,000 ppm	apple puree, 40 days	Cerrutti and Alzamora (1996)
Zygosaccharomyces bailii	20 mM		Fitzgerald (2003)
Zygosaccharomyces rouxii	2,000 ppm	apple puree, 40 days	Cerrutti and Alzamora (1996)
Zygosaccharomyces rouxii	13 mM		Fitzgerald (2003)
Aspergillus flavus	1,300 ppm	pH range 3.5-4.5	Lopez-Malo (2002)
Aspergillus flavus	1,000 ppm	apple and pineapple based agar systems, 2 months	Lopez-Malo *et al*. (1995)
Aspergillus niger	1,500 ppm	apple and pineapple based agar systems, 2 months	Lopez-Malo *et al*. (1995)
Aspergillus ochraceus	1,000 ppm	apple and pineapple based agar systems, 2 months	Lopez-Malo *et al*. (1995)
Aspergillus parasiticus	1,000 ppm	apple and pineapple based agar systems, 2 months	Lopez-Malo *et al*. (1995)

MIC: Minimum Inhibitory Concentration

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One way to reduce vanillin levels whilst retaining the same level of preservation would be to apply it in combination with other antimicrobials or hurdles (Leistner, 2000). The synergistic effects were observed when vanillin and potassium sorbate were used in combination (Lopez-Malo *et al*., 1995). Matamoros-Leon *et al*. (1999) established that with a slight reduction in pH and water activity, 3 mM vanillin in combination with 2 mM potassium sorbate could inhibit the growth of *Penicillium digitatum, Penicillium glabrum* and *Penicillium italicum* for 1 month. Lopez-Malo *et al*. (2005) studied the combined effects of a_w (0.99 or 0.95), pH (4.5 or 3.5) and vanillin concentration on the growth of *Aspergillus flavus* in potato dextrose agar (PDA). Depending on a_w and pH, increase in vanillin concentration slightly reduce the radial growth rate until a critical concentration (1,300 ppm) was reached and the radial growth rate was drastically reduced and mold growth was inhibited. Germination time increased as antimicrobial agent concentration increased and a_w and pH decreased. They also discovered that natural antimicrobials have less pH-dependency than chemical preservatives. Generally, the mold strains investigated were found to be more sensitive to all of the test compounds than the yeast strains. This may indicate that yeast physiology may be better equipped to counteract the antifungal properties of these compounds and with time could overcome them to a greater degree than the molds (Fitzgerald *et al*., 2005).

A number of studies using both prokaryotic and eukaryotic microorganisms have suggested that the inhibitory action of phenolic compounds is due to the presence of the hydroxyl groups (Wendakoon and Sakaguchi, 1995; Aziz *et al*., 1998; Dorman and Deans, 2000 and Ultee *et al*., 2002). The authors reasoned that the hydroxyl groups either reacts with enzyme active sites through the formation of hydrogen bonds (Wendakoon and Sakaguchi, 1995; Aziz *et al*., 1998) or acts as a trans-membrane carrier for monovalent cations (Dorman and Deans, 2000). Fitzgerald (2005) demonstrated that the aldehyde moeity of vanillin plays a key role in its antifungal activity. In addition, the side-group position on the benzene ring also influences this activity. Fitzgerald (2005) found that the two least effective compounds were shown to be phenol and guaiacol, both of which possess a hydroxyl moiety within their structures. Removal of the hydroxyl group from vanillin, 4-hydroxybenzaldehyde, or guaiacol resulted in a slight improvement in activity.

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De Wulf and Thonart (1989) showed that *Saccharomyces cerevisiae* was able to bioconvert vanillin to vanillyl alcohol and acid derivatives. Vanillyl alcohol and vanillic acid have little or no inhibitory effect on the above mentioned yeast. Therefore, aldehyde moiety in the vanillin structure regarded to antimicrobial efficiency. The ability of yeast to bioconvert the vanillin, and hence reduce its concentration could influence the antimicrobial effectiveness of vanillin. However, this conversion process did not occur in culture containing 10-15 mM vanillin (Fitzgerald *et al*., 2003).

2.4 Release of antimicrobial agents from films

The predominant cause of spoilage of many refrigerated foods is microbial growth on the product surface. Incorporation of antimicrobial entities into films are intended to be released onto the surface of a food at a specific rate to control microbial growth. Gradual release may have an advantage over direct incorporation into food. Direct incorporation of antimicrobials may be rapidly lost due to migration into the food bulk or react with food components. For most antimicrobial active packaging concepts, intensive contact between the active material and the food product is required (Devlieghere *et al*., 2004a). The choice of the antimicrobial is often limited by the compatibility of the component with the packaging material or by the heat lability of the component during extrusion (Weng and Hotchkiss, 1993; Han and Floros, 1997). Relatively polar compounds such as sorbate, benzoate and propionate were incompatible with the apolar low density polyethylene (Weng and Hotchkiss, 1993). Acid anhydrides were thought to be more compatible than free acids and their salts because of their lower polarity. Low density polyethylene resin and potassium sorbate powder can be mixed, extruded and pelletized to produce a masterbatch at low temperature to prevent heat decomposition of the potassium sorbate (Han and Floros, 1997).

Many factors affect the mass transfer mechanism of antimicrobial entities from polymeric films. Ouattara *et al*. (2000) reported that diffusion of acetic and propionic acids from chitosan films was found to be unaffected by pH, but decreased as temperature reduced from 24 to 4^{o}C. They explained that the release of acetic and propionic acids from chitosan films immersed in water, occurred when water first

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entered the chitosan matrix and dissolved the acids, thus allowing their subsequent release from the polymer. Therefore, the diffusion rate will be increased with increasing penetration of water into the chitosan films. They found that the acids were completely released from the chitosan matrix in 5-10 minutes. A similar but slower pattern of acid release was observed when chitosan films were applied onto the surfaces of processed meats. However, significantly more acetic acid remained in the chitosan films during storage when the films were applied on bologna than on ham or pastrami. The latter two meat products were characterized by wetter surfaces, suggesting that acid release onto bologna was interrupted earlier, due to liquid volume limitations. Acetic acid diffused out of chitosan films more rapidly than propionic acid in an aqueous medium (Ouattara *et al*., 2000). Its diffusion was not as complete as that of propionic acid when the chitosan films were applied onto processed meats. The release of organic acids from chitosan films is a complex phenomenon affected by many factors, such as electrostatic interactions between acid and polymer molecules (Demarger-Andre and Domard, 1994), ionic osmosis, and structural changes in the polymer induced by the presence of the acids (Narisawa *et al*., 1996). Cellulose based films have been shown to be suitable for slow release of potassium sorbate. Rico-Pena and Torres (1991) showed that potassium sorbate diffusion in methylcellulose film increased as a_w increased from 0.65 to 0.80. Films were not stable for $a_w > 0.80$. Potassium sorbate permeability decreased as pH increased from 3 to 7 at a_w 0.8. Han and Floros (1998) studied the diffusivity of potassium sorbate in American processed and Mozzarella cheeses. They found that the diffusivity of potassium sorbate through American processed cheese was faster than that through Mozzarella cheese because the former contained higher water content. Chen *et al*. (1996) observed a rapid release when working with methylcellulose and chitosan films containing sorbate or benzoate and stated that such a high diffusion rate might in turn reduce the antimicrobial effect of the films for long term storage. Many researchers have studied the diffusion of potassium sorbate from biodegradable films. Choi *et al*. (2005) studied the diffusivity of potassium sorbate in *k*-carrageenan film. It was found that the diffusion followed Fick's law and was unaffected by pH. However, diffusion decreased as temperature decreased. Ozdermir and Floros (2001) studied the diffusivity of potassium sorbate through whey protein film and found

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anomalous or non-Fickian transport. They described the reason for this phenomena was that the film swelled due to countercurrent diffusion (solvent in and potassium sorbate out). Zactiti and Kieckbusch (2005) studied the permeability of potassium sorbate in alginate film. The effect of cross-linking increased the permeability and strength but reduced elongation and solubility. They found that sorbate had a disaggregating effect on the matrix structure, and acted like a plasticizer. Increasing the potassium sorbate concentration resulted in film absorbing a higher amount of water.

Incorporation of hydrophobic compounds into hydrophilic polymers causes structural modifications of the polymer matrix, causing an increase in network tortuosity (Redl *et al*., 1996; Callegarin *et al*., 1997). This impedes the transport of molecules through the network (Papadokostaki *et al*., 1997), and reduces water uptake (Vazquez *et al*., 1997). These effects were successfully exploited by Redl *et al*. (1996), who reduced the diffusion of sorbic acid by 20–50% from whey gluten films, by incorporating beeswax or acetylated monoglyceride into the films. The addition of various fatty acids; lauric, palmitic, stearic, and arachidic acids also has been found to reduce the permeability of MC and HPMC films to potassium sorbate (Vojdani and Torres, 1990).

2.5 Antimicrobial film applications for food

2.5.1 Inhibitory effect of antimicrobial films

Chitosan has been reported to significantly inhibit various meat spoilage bacteria, through its capacity to both bind water and inhibit various enzymes (Young *et al*., 1982), and through its ability to adsorb nutrients normally used by bacteria (Knorr, 1991; Darmadji and Izumimoto, 1994).

Chitosan has been used to coat fruit and vegetables. It can affect product characteristics and microbial loads. Devlieghere *et al*. (2004) reported that chitosan coating of strawberries was applicable while on mixed lettuce the chitosan coating was not applicable due to the development of a bitter taste. Strawberries coated with chitosan tasted bitter on day 0, this abnormality disappeared after 3 days of storage at 7^{o}C. The microbiological load on the chitosan-dipped samples was lower for both products. The antimicrobial effect of chitosan on lettuce disappeared after 4 days of

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storage, while it lasted on strawberries for 12 days. Chitosan treated strawberries had a better texture than untreated strawberries. Savage and Savage (1994) found that apples coated with chitosan had reduced incidence of mold growth over 12 weeks. Cheah and Page (1997) found that Sclerotinia rot of carrots coated with 2 or 4% chitosan was significantly reduced from 88 to 28%.

Many researchers incorporated essential oils or plant extracts into chitosan film but only some applied antimicrobial films to food products (Table 2.2 and 2.3).

Table 2.2 Application of cellulose derivatives and chitosan in antimicrobial food packaging (Cha and Chinnan, 2004).

Biopolymers	Antimicrobial agents	Food	References
Cellulose	Pediocin	Meat	Ming *et al*., 1997
Cellulose based paper	Nisin/Lacticin 3147	Cheese/ham	Scannel *et al*., 2000
HPMC	Ethanol/citric acid/ acetic acid/sorbic acid	Tomato	Zhuang *et al*., 1996
Chitosan	Acetic/propionic acid	Meat	Ouattara *et al*., 2000

Ouattara *et al*. (2000) observed no antibacterial effect when neutralized chitosan film were applied onto the surface of processed meats. This indicates that chitosan alone had no effect on bacterial growth. They demonstrated that addition of cinnamaldehyde to the chitosan matrix generally resulted in greater growth inhibition. Since cinnamaldehyde generally had no marked effect on the rate or extent of acid release. The increased effect was likely due to the additional antibacterial effect of cinnamaldehyde itself (Ouattara *et al*., 1997b; Aureli *et al*., 1992), which diffused from the chitosan matrix during storage and remained at the product surface thereafter. The extent of acetic acid released was limited in chitosan film incorporating 1% lauric acid (Ouattara *et al*., 2000). Since the release of hydrosoluble components from polymer films in which they are incorporated was dependent on the simultaneous entry of water. Inclusion of hydrophobic compounds into hydrophilic chitosan films was expected to reduce diffusion by slowing down film hydration (Vasques *et al*., 1997). They also indicated that the inhibition of indigenous Enterobacteriaceae was more extensive at the surface of bologna than the surface of pastrami because bologna

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Table 2.3 Application of antimicrobial packaging in different food systems (Cha and Chinnan, 2004).

Food product	Antimicrobial agent	Target microorganism
Meat, fish and poultry		
Beef	Pediocin	*Listeria monocytogenes*
	Nisin	*Brochothrix thermosphacta*
	Triclosan	*Brochothrix thermosphacta/Salmonella Typhimurium/Escherichia coli* 0157:H7/ *Bacillus subtilis*
Ground beef	Grape fruit seed extract	*Micrococcus flavus/Escherichia coli/Staphylococcus aureus/ Bacillus subtilis*
Ham	Lacticin 3147 and nisin	*Lactococcus lactis* subsp. *lactis/Listeria innocua/ Staphylococcus aureus*
Poultry	Nisin	*Salmonella typhimurium*
Ham/bologna/pastrami	Acetic acid and propionic acid	*Lactobacillus askei/Serratia liquefaciens*
Vacuum beef carcass	Nisin	*Lactobacillus helveticus/Brochothrix thermosphacta*
Fresh broiler skin	Nisin	*Salmonella typhimurium*
Vegetable products		
Strawberry	Potassium sorbate and citric acid	Aerobic mesophilic/psychrotrophic/ molds/yeast/coliforms
Tomato	Citric acid, acetic acid, sorbic acid and ethanol	*Salmonella montevideo*
Lettuce/soybean sprouts	Grape fruit seed extract	*Escherichia coli/Staphylococcus aureus*

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Table 2.3 – continued.

Food product	Antimicrobial agent	Target microorganism
Milk and dairy products		
Skim milk	Nisin	*Lactobacillus curvatus*
Cheddar cheese	Lacticin 3147 and nisin	*Lactococcus lactis* subsp. *Lactis/Listeria innocua/ Staphylococcus aureus*
Cheese	Imazalil	*Penicillium sp./Aspergillus toxicarius*

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contains efficient water binding agents, and so exudes little water during storage. As a result, release of acetic acid onto the surface was more beneficial. Zivanovic *et al*. (2005) placed chitosan film and chitosan-oregano essential oil film in contact with inoculated bologna samples and stored 5 days at 10°C. Pure chitosan films reduced *Listeria monocytogenes* by 2 logs, whereas the films with 1% and 2% oregano oil decreased the number of *Listeria monocytogenes* by 3.6 to 4 logs and *Escherichia coli* by 3 logs. Oregano oil was more effective against Gram-positive *Listeria monocytogenes* than against Gram-negative *Escherichia coli.* Pure chitosan film, with no essential oil, had no inhibitory effect against the bacteria tested in inoculated agar plates. The inoculum in this experiment was 10^6 CFU/petri dish, whereas others used much lower inoculum (10^2 CFU/petri dish) for similar experiments (Coma *et al*., 2002). Thus, the high number of bacteria may have exceeded the inhibition activity of chitosan. The inhibitory effect of oregano oil which was incorporated into the chitosan film was lower than that of pure essential oil. The possible reason for the decrease in activity of the essential oil in the chitosan film may have been due to partial loss of highly volatile compounds from the essential oil during film preparation. Also there may have been due to slower release of active compounds from the chitosan film than from the cellulose filter paper. Lee *et al*. (1998) incorporated 1% grapefruit seed extract into low density polyethylene film and used it to package curled lettuce. It was found that the growth rates of aerobic bacteria and yeast decreased. Pranoto *et al*. (2005) incorporated garlic oil into chitosan film and compared its preservative effect with conventional food preservatives: potassium sorbate and bacteriocin (nisin). The activity of the antimicrobial films was tested against food pathogens, *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Listeria monocytogenes* and *Bacillus cereus*. They found that the chitosan film had no inhibitory effect. Incorporation of 100 µl of garlic oil/g, 100 mg potassium sorbate/g or nisin at 51,000 IU/g of chitosan had antimicrobial activity against *Staphylococcus aureus, Listeria monocytogenes,* and *Bacillus cereus*.

The inhibitory effect and other properties of cellulose based antimicrobial films were investigated by Coma *et al*. (2001). Nisin and stearic acid were incorporated into a HPMC based film. Fifteen percent (w/w HPMC) of stearic acid improved film moisture barrier. However, mechanical resistance and antimicrobial activity on *Listeria monocytogenes* and *Staphylococcus aureus* were both reduced due to interactions between the cationic nisin and the anionic stearic acid which decreased

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nisin desorption from the film. The anionic effect of stearic acid is pH dependent. At pH 6.1, stearic acid was preferentially negatively charged. Adjusting the pH to 3.0 prevented any interaction between stearic acid and nisin, and thus a film of high inhibitory activity was created. Cha *et al*. (2003) incorporated nisin into methylcellulose film and reported that the higher amount of the methoxyl group resulted in a strong bond with nisin, which reduced antimicrobial activity. They found that the antimicrobial activity of nisin was greater in chitosan/methylcellulose film even though chitosan was proven to have no inhibitory effect on the microorganisms studied. They explained that nisin contains positive charges and the viscosity of chitosan/methylcellulose films decreases as a result of increase in the concentration of electrolytes in the film. Therefore, the addition of nisin to chitosan/methylcellulose films results in high release rates.

2.5.2 Film properties after incorporation of antimicrobial agents

The incorporation of antimicrobials into films significantly changed the functional characteristics of the packaging materials. Incorporation of essential oil into chitosan film reduced water vapor permeability (Yanishlieva *et al*., 1999; Botsoglou *et al*., 2002). Zivanovic *et al*. (2005) found that the addition of oregano oil into the chitosan film decreased water vapor permeability, puncture and tensile strength, but increased elasticity of the films. Essential oil also resulted in a thicker and more opaque film. The increased film opacity is probably due to light scattering from lipid droplets, which were distributed throughout the polymer network after the film formed (Debeaufort *et al*., 2000). Pranoto *et al*. (2005) reported that addition of garlic oil up to levels of at least 100 µl/g, potassium sorbate at 100 mg/g and nisin at 51,000 IU/g of chitosan into films were physically acceptable in terms of appearance, mechanical and physical properties. A greater reduction of tensile strength and elongation was caused by incorporation of potassium sorbate and nisin. Garlic oil did not affect WVP while potassium sorbate 150 mg/g and nisin 153,000 IU/g of chitosan significantly increased WVP. The antimicrobial agents contributed to an extension of the intermolecular network and a loosening of the structure. This enhanced the ability of moisture to pass through the films and increased the WVP values of the films. However, this did not occur when chitosan film incorporated with garlic oil which is a hydrophobic material (Ross *et al*., 2001). FTIR showed that garlic oil components have no interaction with the functional groups of chitosan.

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