CHARACTERIZATION AND ANTIMICROBIAL ANALYSIS OF CHITOSAN COMPOSITE BIODEGRADABLE FILMS WITH ADDITION OF LEMON GRASS ESSENTIAL OIL

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ABSTRACT

The increasing demand in food safety, quality, convenience and environmental concerns associated with the handling of plastic waste has emphasized the importance in developing biodegradable and edible films from natural polymers, such as chitosan. Starch-based film is considered an economical material for antimicrobial packaging. This study aimed at the development of packaging based on chitosan-tapioca starch incorporated with lemon grass essential oil as antimicrobial agents. In this research, there are three samples were prepared. Sample A containing starch-chitosan as control film, sample B containing starch-chitosan with the addition of gelatin and sample C containing starch-chitosan based film incorporated with lemon grass essential oil. For the antimicrobial analysis, all the samples were tested on *B. substilis* and *E. coli*. Inhibition of bacterial growth was examined using two methods which are zone inhibition assay and liquid culture test. From the observations, sample C exhibited a wide clear inhibitory zone rather than samples A and B. From the liquid culture test, the sample C clearly demonstrated a better inhibition against *B. substilis* than *E. coli*. Incorporation of lemon grass essential oils also led to an improvement in several films properties in terms of morphology, chemical composition and thermal properties. The result from TGA and DSC has shown the higher thermal and melting temperature possesses by the sample C and slightly miscible and compatible with the starch-chitosan composition. The addition of lemon grass essential oil proves that the film is better to film based on starch-chitosan only. Lemon grass essential oil will actually improve the performance of the chitosan composite biodegradable film as tested in this research. As a conclusion, starch-chitosan composite biodegradable film has the potential to be use for food packaging.

ABSTRAK

Permintaan yang tinggi terhadap keselamatan makanan, kualiti dan masalah persekitaran alam yang berkaitan dengan pengurusan pembuangan plastik telah menekankan tentang kepentingan mencipta filem yang boleh dibiodegradasikan daripada polimer semulajadi seperti chitosan. Filem yang diperbuat daripada kanji dan chitosan ini sesuai sebagai sumber ekonomi untuk pembungkusan antimikrob. Kajian ini menekankan tentang pembungkusan yang diperbuat daripada kanji dan chitosan dan dicampurkan dengan minyak pati serai wangi. Dalam kajian ini, tiga sampel telah disediakan. Sampel A mengandungi kanji dan chitosan sahaja yang bertindak sebagai kontrol filem, sampel B mengandungi kanji dan chitosan ditambah dengan gelatin dan sampel C mengandungi kanji dan chitosan ditambah dengan minyak pati serai wangi. Untuk analisis antimikrob, kesemua sampel telah diuji terhadap *B. substilis* dan *E. coli*. Perencatan terhadap pertumbuhan bacteria telah di uji melalui dua cara iaitu *zone inhibition assay* dan *liquid culture test.* Sampel C menunjukkan kawasan zon halangan yang terbesar berbanding dengan sampel A dan B. Daripada analisis pembiakan bakteria, sampel C telah menunjukkan halangan yang tinggi terhadap *B. substilis* berbanding dengan *E. coli*. Penambahan minyak pati serai wangi telah memberi pembaharuan dari segi morfologi, komposisi kimia dan sifat terma. Keputusan dari TGA dan DSC telah menunjukkan ketahanan haba dan suhu peleburan haba yang tinggi yang tinggi dan sedikit *miscible* dan serasi dengan komposisi kanji dan chitosan. Minyak pati serai wangi akan meningkatkan kualiti chitosan komposit biodegradasi filem sebagaimana yang telah diuji dalam kajian ini. Kesimpulannya, filem komposit boleh biodegradasi yang diperbuat daripada kanji dan chitosan mempunyai potensi untuk digunakan sebagai pembungkus makanan.

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LIST OF SYMBOLS

TPS	Thermoplastic starch
PEG	Polyethylene glycol
SEM	Scanning electron microscope
FTIR	Fourier transforms infrared spectroscopy
TGA	Thermo gravimetric analysis
DSC	Differential scanning calorimeter
EO	Essential oil
% v/v	Percent volume per volume
λ	Wavelength
β	Heating rate
T	Temperature
T_M	Melting temperature

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Edible films and coatings have been particularly considered in food preservation, because of their capability in improving global food quality (Franssen and Krochta, 2003; Franssen *et al*., 2002; Guilbert and Biquet, 1996; Greener Donhowe and Fennema, 1994). The films can be used to cover food surfaces, separate incompatible zones and ingredients, form a barrier against oxygen, aroma, oil and moisture or perform as pouches or wraps. Among other important features, they can be used as carriers of functional agents, as antioxidants or antimicrobials, and to improve appearance and handling. Film production by natural and abundant biodegradable polymeric materials as cellulose, gums, starches or proteins, is also convenient due to the lower environmental consequences compared with common synthetic plastic materials (Cutter, 2006).

Edible and biodegradable films are always not meant to totally replace the synthetic packaging films (Krochta and Johnston, 1997) though it is one of the most effective methods of maintaining food quality. Usually film-forming substances are based on proteins, polysaccharides lipids and resins or a combination of these (Greener-Donhowe and Fennema, 1994). Chitosan has been found to be nontoxic, biodegradable, bio-functional, bio-compatible in addition to having antimicrobial characteristics

(Darmadji and Izumimoto, 1994; Jayakumar *et al*., 2007; Jayakumar *et al*., 2005; Jayakumar *et al*., 2006; Jongrittiporn *et al*., 2001; Wang, 1992). As compared with other bio-based food packaging materials, chitosan has the advantage of being able to incorporate functional substances such as minerals or vitamins and possesses antibacterial activity (Chen *et al*., 2002; Jeon *et al*., 2002; Moller *et al*., 2004). In view of these qualities, chitosan films have been used as a packaging material for the quality preservation of a variety of food (Park and Zhao, 2004; Suyatma *et al*., 2005; Tsai and Su, 1999; Wu *et al*., 2005). Recently, a chitosan-starch film has been prepared using microwave treatment which may find potential application in food packaging. A wide variety of chitosan based antimicrobial films have recently been well documented (Tripathi *et al*., 2008).

Antimicrobial packaging has been touted as a major focus in the next generation of active packaging (Brody, 2001). Antimicrobial packaging is the packaging system that is able to kill or inhibit spoilage and pathogenic microorganisms that are contaminating foods. When the packaging system acquires antimicrobial activity, the packaging system limits or prevents microbial growth by extending the lag period and reducing the growth rate or decreases live counts of microorganisms (Han, 2000). Antimicrobial packaging can extend the food shelf-life, thus improving the quality of the food. Interest in antimicrobial packaging films has increased in recent years due to a concern over the risk of food borne illness, desire for extended food shelf life, and advances in the technology of film production. Slowing the growth of spoilage bacteria will reduce the losses of product to spoilage and extend shelf life. Reduction of pathogen growth will reduce the risk of food borne illness caused by those products (Dawson *et al.,* 2002). It can effectively control the microbial contamination of various solid and semisolid foodstuffs by inhibiting the growth of microorganisms on the surface of the food, which normally comes into direct contact with the packaging material. Antimicrobial function of the packaging system can be achieved by incorporating active substances into the packaging system by various ways (Han, 2003). The antimicrobial packaging is conducted by (1) the addition of antimicrobial containing sachets or pads into food packages; (2) the coating, immobilization or direct

incorporation of antimicrobials into food packaging materials or (3) the use of packaging materials that are inherently antimicrobial (Appendini and Hotchkiss, 2002). One of the examples of antimicrobial agents is lemon grass, besides Onawunmi *et al.* (1984) reported that the antimicrobial activity of lemongrass oil is related to high amounts of 1,8-cincole (>30%), geranial (>30%), and neral (>20%). However, citral isomers (neral, 32.2%, geranial, 41.28%) are the most abundant compounds in lemongrass oil as reported by (Choi *et al.,* 2000). These components individually showed antibacterial action on gram negative and gram-positive organisms (Onawunmi *et al.*, 1984).

1.2   Problem Statement

The rise in environmental consciousness in recent decades has included a focus on household waste. Food packaging has come to symbolize the issue of waste which formerly known as non degradable. It has expanded rapidly in recent times and perhaps most important of all, food packaging feels wasteful: used once and then promptly discarded, it seems like only a temporary presence in our lives as it rushes from factory to landfill. Nowadays, about 150 million tons of plastic are produced annually all over the world, and the production and consumption continue to increase (Parra *et al.,* 2004). Most of these plastics are crude oil based. In addition, handling of plastic waste associated with serious environmental pollution problem due to waste disposal and undegraded polymers. Therefore, the use of agricultural biopolymers that are easily biodegradable not only would solve these problems, but would also provide a potential new use for surplus farm production (Okada, 2002; Pavlath and Robertson, 1999; Scott, 2000). Because of the environmental concerns and technological problems such as denaturing effects of thermal polymer processing methods, extrusion and injection molding, the incorporation of bio-preservatives into biodegradable films is more suitable than incorporation into plastic films (Appendini and Hotchkiss, 2002; Han, 2000; Suppakul *et al.,* 2003)

Over the last few years, considerable research has been conducted to develop and apply bio-based polymers made from a variety of agricultural commodities and or wastes of food product industrialization (Cutter, 2006; Guilbert and Biquet, 1996). Among various degradable membrane materials, chitosan has attracted considerable attention for its unique properties, the most important one of which is abundant commercial supplies and antibacterial properties. Although chitosan membranes are highly impermeable to oxygen, they have relatively poor water vapor barrier characteristics due to its excellent hydrophilicity, which is not favorable for the usage as artificial skins (Willfor et al., 2008). But adding plasticizers such as glycerol had negative effects on barrier properties in spite of positive effects on mechanical properties (Srinivasa et al., 2007). Among efforts have been done to improve water barrier capability of chitosan (weakening its hydrophilic property) was blending chitosan with some hydrophobic materials had attached most interest. In order to improve the physical and functional properties of chitosan films, blending with other biopolymers such as starch. Starch based films have been particularly considered for the reason that they exhibit physical characteristics similar to synthetic polymers: transparent, odorless, tasteless, semi-permeable to CO_2 and resistant to O_2 passage (Nísperos and Carriedo, 1994).

Throughout the years, many researchers- have been done to improve the performance of biodegradable film in the food packaging area. A widespread trend worldwide is the movement towards natural food products. In effort to meet this demand, there has been increased interest in the food industry in using antimicrobial preservatives that are perceived as more natural. Future work will focus on the use of biologically active derived antimicrobial compounds bound to biopolymers. However many natural antimicrobials have a limited spectrum of activity and are effective only at very high concentrations. The need for new antimicrobials with wide spectrum activity and low toxicity will increase. A possible solution may be using combinations of antimicrobials (Sofos et $al.,$ 1998). Instead of concentrating on development of new antimicrobial, it could be more practical to combine the antimicrobial agents that already being researched From all the points above, this research are emphasize on

make a biodegradable film from chitosan blend with tapioca starch to enhances its mechanical and functional properties with additional of lemon grass essential oil as antimicrobial agent.

1.3   Research Objectives

The objectives of this study are listed as following:

a. To fabricate a chitosan composite biodegradable film with combination of gelatin and lemon grass essential oils.

b. To analyze the fabricated films in terms of morphology, physical and chemical properties.

c. To perform the antimicrobial analysis against bacteria strain.

1.4   Scope of Study

The scopes of this study are listed as following:

a. To fabricate a chitosan composite biodegradable film with addition of tapioca starch, plasticizer, gelatin and lemon grass essential oils.

b. To analyze the fabricated films in term of antimicrobial activity using zone inhibition assay and liquid culture test against *Bacillus Subtilis* and *Escherichia coli.*

c. To characterize the fabricated films in terms of morphology, physical and chemical properties using various analysis method:

    i.   Scanning Electron Microscopy (SEM)

    ii.   Fourier Transform Infrared Spectroscopy (FTIR)

    iii.   Thermo Gravimetric Analysis (TGA)

    iv.   Differential Scanning Calorimeter (DSC)

CHAPTER 2

LITERATURE REVIEW

2.1   Composite Biodegradable Film

Bio-composites (biodegradable composites) consist of biodegradable polymers as the matrix material and biodegradable fillers, usually bio-fibers. Since both components are biodegradable, the composite as the integral part is also expected to be biodegradable (Mohant *et al*., 2000c).

Another important bio-composite category is based on agro-polymers matrixes, mainly focused on starchy materials. Plasticized starch, called thermoplastic starch (TPS) is obtained after disruption and plasticization of native starch, with water and plasticizer by applying thermo mechanical energy in a continuous extrusion process. Unfortunately, TPS shows some drawbacks such as a strong hydrophilic character, rather poor mechanical properties compared to conventional polymers and an important post-processing variation of the properties. TPS properties reach equilibrium only after several weeks. To improve these material weaknesses, TPS is usually associated with others compounds (Dufresne and Vignon, 1998; Dufresne *et al*., 2000), bleached leaf wood fibers (Funke *et al*., 1998; Ave´rous *et al*., 2001). Most of these authors have shown that between both polysaccharides, a high compatibility occurs. They have found high improvements of the performances in terms of and impact tests results, which are

in part linked to usual matrix reinforcement (Bledzki and Gassan, 1999). Another part of the mechanical properties increase is brought by the inter-relations fibre-matrix. The main attributes are higher moduli (Dufresne and Vignon, 1998; Dufresne *et al.*, 2000; Funke *et al.*, 1998; Ave´rous *et al.*, 2001; Curvelo *et al.*, 2001), reduced water sensitivity due to fibre–matrix interactions and to the higher hydrophobic character of the cellulose, which is linked to its high cristallinity (Funke *et al.*, 1998; Ave´rous *et al.*, 2001; Curvelo *et al.*, 2001). Fibres addition induces variation of properties, due to the formation of a 3D network between the different carbohydrates, through hydrogen bonds.

2.1.2   Biodegradable Film from Starch and Chitosan Blend

Previous study showed that cassava starch can readily be cast into films. However, the cassava starch film is brittle and weak leading to inadequate mechanical properties. Overcoming the brittleness of the film can be accomplished by adding plasticizers. Common plasticizers used for starch films preparation are water, glycerol, sorbitol, and other low-molecular weight polyhydroxy compounds (Rindlav *et al.*, 1998). Water is an excellent plasticizer; however, it has some disadvantages since water content varies with humidity. At low humidity there are problems with brittleness and at high humidity with softness. Glycerol and sorbitol are widely used as plasticizers because of their stability and edibility. Addition of plasticizers makes the brittle films more flexible, but also less strong. This problem has led to the development of mechanical properties of cassava starch film. Blending (Chandra and Rustgi, 1998) or laminating (Coffin and Fishman, 1993) with other materials could improve the disadvantages. The scope of films made with starch combined with other polysaccharides was widened to include chitosan for several reasons. First, chitosan is a biopolymer, obtained by N-deacetylation of chitin, which is the second most abundant polysaccharide on the earth after cellulose (Arvanitoyannis *et al.*, 1998). It is commercially available from a stable renewable source, that is, shellfish waste (shrimp and crab shells) of the sea-food industry. Second, chitosan forms good films and

membranes. Chitosan films that were clean, tough, flexible and good oxygen barriers were formed by solution casting (Jeon *et al*., 2002). Composited films from chitosan and cellulose have been made by casting dispersions on the steel or chrome plates at elevated temperatures from 70 to 100˚C (Nishiyama, 1993). Some of these films contained glycerol and had good tensile strength. They were readily biodegradable either in sea water or in soil. Third, the cationic properties of chitosan offer the film-maker an opportunity to take advantage of electrostatic interactions with other anionic polysaccharides. In addition, chitosan possesses useful properties such as biodegradability, biocompatibility (Sashiwa *et al*., 2003), and non-toxicity leading to extensively use over a wide range of applications. Chitosan film has a potential to be employed as packaging, particularly as an edible packaging. This is due to its excellent oxygen and carbon dioxide barrier properties and interesting antimicrobial properties.

2.2   Chitosan

2.2.1   History of Chitosan

The history of chitosan can be traced back to 1811 when chitin was first discovered by Braconnot, a professor of the natural history in France. According to some researches, while Braconnot was conducting research on mushrooms, he isolated what was later to be called chitin (Dutta, 2009).

Twenty years later, there was a man who wrote an article on insects in which he noted that similar substance was present in the structure of insects as well as the structure of plants. He then called this astounding substance as chitin (Dutta, 2009).

Basically, the name chitin is derived from Greek, meaning tunic or envelope. The concept was further known in 1843 when Lassaigne demonstrated the presence of nitrogen in chitin (Franciele, 2009).

Following the discovery of chitin, the name chitosan emerged in the scene. It was first discovered by Rouget while experimenting with chitin. Rouget observed that the compound of chitin could be manipulated through chemical and temperature treatments for it to become soluble. Then, it was in 1878 when Ledderhose identified chitin to be made of glucosamine and acetic acid. It was not actually until 1894 that Hoppe-Seyler named the tailored chitin, chitosan (Franciele, 2009).

During the early 20th century, several researches took chitosan as their subject of study. They then involved sources of chitin, including crab shells and fungai. It was the work of Rammelberg in the 1930s that led to the confirmation on the identity of chitosan from these sources. It was also noted that by hydrolyzing chitin in several ways, it was determined by experts that chitin is a polysaccharide of glucosamine (Cholwasa, 2006).

During the 1950s, the use of x-ray analysis had advanced the study of the incidence of chitin or chitosan in fungi. However, it is only the most advanced technologies that proved the most reliable in accepting the existence of chitin as well as cellulose in the cell walls (Fujun, 2009).

2.2.2 Properties of Chitosan

Chitosan, b-1, 4 linked glucosamine and N-acetyl glucosamine, is prepared by deacetylation of chitin. Chitosan has been proved to be nontoxic, biodegradable, biofunctional, biocompatible and have antimicrobial characteristics (Wang, 1992; Darmadji and Izumimoto, 1994; Jongrittiporn *et al*., 2001).The reasons for chitosan

addition in edible films are the good film forming and mechanical properties, no toxicity, biodegradability, relative more hydrophobic nature that could provide higher moisture barrier and water resistance (Bangyekan *et al.*, 2006; Mathew and Abraham, 2008).
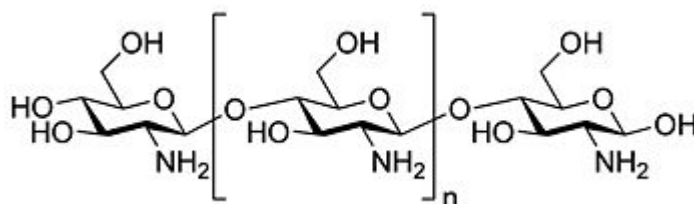

Figure 2.1: Structural molecule of chitosan

2.3   Tapioca/Cassava Starch

Cassava or tapioca is one of the economically important crops in Thailand and the cheapest raw material of starch production. Structurally, cassava starch consists of two types of molecules: amylose, a substantially linear polymer with a molecular weight of about 105; and amylopectin, a highly branched polymer with very high molecular weight of about 107. The approximate 17% of amylose content is responsible for strong film forming characteristics (Rindlav *et al.*, 1998).
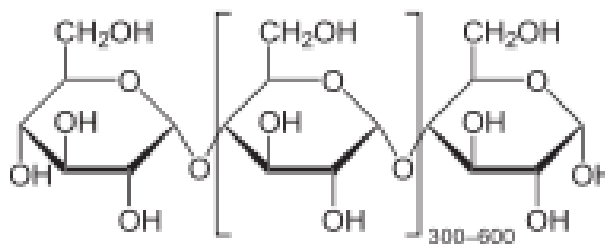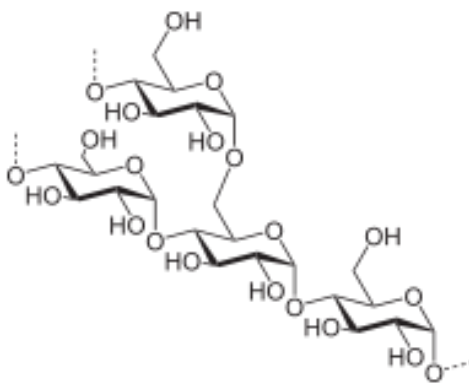

Figure 2.2: Amylose molecule

Figure 2.3: Amylopectin molecule

Tapioca starch, naturally or modified, is increasing its utility in food industry because it has some inherent properties that are demanded. The preferred properties of cassava starch include: high transparency, determining suitability for developing sauces for ready-to-eat foods; high resistance to acidity, allowing its use for acid-based sauces and jams. It is also applicable for desserts, puddings, soups, fillings and gums due to its high viscosity. As an alternative starch it could replace traditional starches because it is also a lower cost option. Unfortunately it is not easy to replace starches traditionally used because it is difficult to overcome the strong links that exist between producers, starch manufacturers and food industries that utilize this polysaccharide in main importing countries of Europe and North America (Fao, 2004). For these reasons, it is interesting to explore the possibility of developing new products based on tapioca starch, to research about alternative applications and to solve the lack of information about its role as alternative source of starch tending to help to increase its added value (Silvia, 2006)

The formation of starch edible film involves gelatinization of starch granules by heating in excess water. This procedure results in granule swelling and disruption as well leaching of soluble components (amylose) from the granule. A viscous mass is obtained and it consists of a continuous phase constituted basically by solubilized amylase and a discontinuous phase of remnant granules, mainly based on amylopectin

(Zobel, 1994). Cooling of the hot paste, results in a visco-elastic gel. The formation of the junction zones (polymer molecules joined by covalent bonds, hydrogen bonding and/or Van der Waal forces) of a gel can be considered to be the first stage of an attempt by starch molecules to crystallize. The collective processes that take part in the reduction of the solubility of dissolved starch are called retrogradation and involve the two constituent polymers, amylose and amylopectin, with amylase undergoing retrogradation at a much more rapid rate than does amylopectin. The rate of retrogradation depends on several variables, including the molecular ratio of amylase to amylopectin, structures of the amylose and amylopectin molecules (botanic source of the starch), starch concentration, presence and concentration of other ingredients, such as surfactants, lipids and salts, processing conditions like temperature and shear (Miller and Whistler, 1996). Gelation and retrogradation can be interpreted as the result of double helices forming a network of physically cross-linked molecules. As initial juncture points grow into helical segments and then aggregate into A–B-type crystallites, gels or retrograded materials become more rigid and difficult to disperse (Zobel, 1994).

2.4   Polyethylene Glycol 400 (PEG 400)

Polyethylene Glycol 400 (PEG 400) is a low molecular weight grade of polyethylene glycol. It is a clear, colorless and viscous liquid. Due in part to its low toxicity, PEG 400 is widely used in a variety of pharmaceutical formulations.

PEG 400 is strongly hydrophilic, the partition coefficient of polyethylene glycol 414 between hexane and water is 0.000015 ($\log P$ = - 4.8), indicating that when polyethylene glycol 414 is mixed with water and hexane, there are only 1.5 parts of polyethylene glycol 414 in the hexane layer per 100,000 parts of polyethylene glycol 414 in the water layer. PEG 400 is soluble in water, acetone, alcohols, benzene,

glycerin, glycols, and aromatic hydrocarbons and is slightly soluble in aliphatic hydrocarbons (Renoulf *et al.*, 2005).

The higher concentration of polyethylene glycol in the composite films, the more higher the water vapour transmission rate will be, it is due to progressive film plasticization which is associated with modification of the hydrophilic character of polylactic acid film. The PEG, thus decrease the material cohesion by creating intermolecular spaces and increasing water molecule diffusion coefficient or the easier separation of PEG with the amorphous phase of polylactic acid (Renoulf *et al.*, 2005).

The content of PEG will also reduce the rigidity and the brittleness of materials, thus improving their mechanical properties and their recovery. It is due to hydrolytic reactions by water absorption (Renoulf *et al.*, 2005).

2.5   Food-borne Bacteria

Preservation of foods has, since the beginning of mankind, been necessary for our survival. The preservation techniques used in early days relied without any understanding of the microbiology on inactivation of the spoiling microorganisms through drying, salting, heating or fermentation. These methods are still used today, albeit using less and less preservation and combining various lightly preservation procedures to inhibit growth of microorganisms. Spoilage is characterized by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. This may be physical damage, chemical changes (oxidation, color changes) or appearance of off-flavors and off-odors resulting from microbial growth and metabolism in the product. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), as text-ural changes (degradation of polymers) or as off-odors and off-flavors. Despite chill chains, chemical

preservatives and a much better understanding of microbial food spoilage, it has been estimated that 25% of all foods produced globally is lost post harvest or post slaughter due to microbial spoilage (Hormazabal, 2007).

Food-borne diseases are still a major concern in some developing countries. Due to the world awareness on chemical preservatives the food industry is now reflected by the consumer opinions for safer additives and thus focusing on natural preservatives (Dillon and Board, 1994). Spices are herbal products which have been safely used by people around the world to impart desirable flavors and aromas to the local foods. It looks that there has been a natural selection for spices as these products are mainly originated from plants grown in the tropical regions with wide distribution of food-borne bacteria. Several of these spices and their essential oil extracts have been reported to possessed antimicrobial activities including garlic, savory, basil, laurel, mint, cumin, onion, sumac, thyme and lemon grass (Arora and Kaur, 1999; Delgado *et al.,* 2004; Nasar *et al.,* 2004; Ozcan and Erkmen, 2001).

2.5.1   Gram Positive Bacteria

Identification of the Gram-positive bacteria has traditionally relied on the thick peptidoglycan cell wall found in most members of this group (Bone and Balkwill, 1998; Buck, 1992; Doetsch, 1991). However, some species those are Gram-positive by phylogenetic criteria (Woese, 1997) which are lack typical cell walls and have variable or negative Gram stain reactions (Stackebrandt *et al.,* 1995). Branched-chain fatty acids have been considered as a marker for actinomycetes and Gram-positive bacteria in natural samples (Zelles and Bai, 1994), but branched-chain fatty acids are also produced by several other bacterial groups (Zelles *et al.,* 1995) in gram positive bacteria.

2.5.1.1 *Bacillus Subtilis (B. Subtilis)*

Bacillus subtilis, known as grass bacillus, is a Gram-positive, catalase-positive bacterium commonly found in soil (Madigan, 2005). A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct (Nakano, 1998).

In 1835, the bacterium was originally named *Vibrio subtilis* by Christian Gottfried Ehrenberg (Ehrenberg, 1835), and renamed *Bacillus subtilis* by Ferdinand Cohn in 1872 (Nakamura, 1997). Cultures of *B. subtilis* were used throughout the 1950s as an alternative medicine due to the immune stimulatory effects of its cell matter, which upon digestion has been found to significantly stimulate broad spectrum immune activity including activation of specific antibody IgM, IgG and IgA secretion (Cohn, 1992) and release of CpG dinucleotides inducing INF A/Y producing activity of Leukocytes and Cytokines important in the development of cytotoxicity towards tumor cells (Shylakhovenko, 2003).

B. subtilis is not considered a human pathogen; it may contaminate food but rarely causes food poisoning (Ryan, 2004). *B. subtilis* produces the proteolytic enzyme subtilisin. *B. subtilis* spores can survive the extreme heating that is often used to cook food, and it is responsible for causing ropiness which is a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough.

The significance of the *Bacillus subtilis* group in food borne disease remains uncertain (Kramer and Gilbert, 1999). The identification of an enteric pathogen is dependent on demonstration of known enterotoxin genes and the functional activity of the toxins and direct proof of enterotoxigenic activity relies on animal models of