Incorporation of Natural Antimicrobial Agents into Starch-Based Material for Food Packaging

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Declaration

I, Kuorwel Kuai Kuorwel, declare that the PhD thesis entitled “Incorporation of Natural Antimicrobial Agents into Starch-Based Material for Food Packaging” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

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Abstract

Antimicrobial (AM) films comprising of thermoplastic starch (TPS) and a commercial aliphatic polyester blended with thermoplastic starch (APTPS) were prepared and their properties, release of AM agents, and AM efficacy was studied in laboratory media and on a real foodstuff (Cheddar cheese). The AM films were prepared using two techniques: (i) direct incorporation of AM agents (carvacrol, linalool or thymol) into TPS films *via* a compression moulding technique; and (ii) by application of a coating containing the AM agents onto the commercial APTPS films.

The mechanical performance of the TPS and APTPS films was investigated after exposure to mixtures of water in isopropanol, water in ethanol and water in glycerol of various compositions and at different levels of relative humidity (RH) conditioned and evaluated at a temperature of 20 ± 1°C. Considerable absorption of moisture, particularly at high RH levels, was found for the TPS films and the mechanical properties changed significantly. At equilibrium RH level of 75%, the tensile strength declined by up to 66% and the Young's modulus and elongation at break decreased by up to 73% and 76% respectively compared with RH of 0%. It is therefore concluded that these films are suitable for packaging of foodstuffs that have a water activity lower than 0.75. Conversely, the APTPS films were stable at all moisture contents and RH levels tested and are thus suitable for packaging of most foods. The effects of AM agents on the permeability, physico-mechanical, optical and thermal properties of the TPS films were also investigated. There was no significant effect of the AM agents on any of these properties when compared to permeability, physico-mechanical, optical and thermal properties of control TPS films.

In order to assess the release of the AM agents from the prepared TPS and APTPS films, migration studies into a food simulant (isooctane) were conducted as a function of temperature. For both types of AM films studied, the AM agents were effectively released into isooctane and the rate became more rapid with increasing temperature. The release of the AM agents was according to first-order kinetics and also followed established short-term and long-term release models. For the compression-moulded TPS films, the diffusion coefficients increased by 100%, 104% and 148% for linalool, carvacrol and thymol respectively when the temperature was increased from 15 to 35°C. Over the same temperature range, the diffusion coefficients from the coating matrix on the APTPS films increased by 84%, 295% and 126% for linalool, carvacrol and thymol respectively. The results suggest that the selected AM agents are suitable for packaging of foods where both short-term and long-term AM activity is required for shelf life extension.

The AM activity of APTPS films coated with AM agents against *Staphylococcus aureus, Saccharomyces cerevisiae* and *Aspergillus niger in vitro* and/or inoculated on the surface of Cheddar cheese was investigated. In solid media, all the AM films demonstrated a positive inhibitory effect against each of the microorganisms. At the highest level of 2.38% (w/w) linalool, carvacrol and thymol, an average zone of inhibition of 18.3 ± 1.0, 21.9 ± 1.7 and 23.8 ± 1.8 mm was observed for *S. aureus* and an average zone of inhibition of 11.2 ± 0.3, 12.2 ± 0.4 and 13.2 ± 0.3 mm was observed for *S. cerevisiae* respectively. In the case of the control film, no inhibitory effect was observed against *S. aureus*, *S. cerevisiae* and *A. niger* on solid media. All the AM films effectively inhibited the growth of all the microorganisms tested on the surface of

Cheddar cheese. The AM films coated with *ca.* 2.38% (w/w) linalool; carvacrol or thymol reduced the population of *A. niger* on the surface of Cheddar cheese by 37%, 41% and 46% respectively after 35 days of storage at 15˚C. The AM activity invariably followed the order of thymol > carvacrol > linalool for the studies conducted *in vitro* and on Cheddar cheese. The results suggest that starch-based film coated with AM agents have the potential for use as packaging systems for real foods such as Cheddar cheese.

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Abbreviations and Nomenclature

AM	Antimicrobial
ANOVA	Analysis of variance
AP	Active Packaging
APTPS	Aliphatic polyesters thermoplastic starch
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
CFU	Colony forming units
CTA	Cellulose triacetate
DSC	Differential scanning calorimetry
EOs	Essential oil
EU	European Union
EVA	Ethylene vinyl acetate
EVOH	Ethylene vinyl alcohol
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FID	Flame ionization detector
FTIR	Fourier Transform Infrared
GC	Gas chromatograpy
GCMS	Gas chromatography mass spectrometer
GLM	General linear model
GRAS	Generally Recognized as Safe
GFSE	Grapefruit seed extract
HDPE	High-density polyethylene
HPMC	Hydroxypropylmethyl cellulose
LB	Lactic acid bacteria

LDPE	Low-density polyethylene
LLDPE	Linear low density polyethylene
MAP	Modified atmosphere packaging
MC	Methylcellulose
MIC	Minimum inhibitory concentration
NCTC	The National Collection of Type Cultures
NIR	Near Infrared
OEO	Essential oil of oregano
PDA	Potato dextrose agar
PE	Polyethylene
PEG	Polyethylene glycol
PET	Polyethylene-terephthalate
PLA	Poly (lactic acid)
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
PVDC	Polyvinylidene chloride
PVOH	Polyvinyl alcohol
RH	Relative humidity
SAS	Statistical Analysis Software
SC	Sodium caseinate
SEM	Scanning electron microscopy
SPI	Soy protein isolate
TEO	Thyme essential oil
TPS	Thermoplastic starch

UNSW	University of New South Wales
VP	Vacuum packaging
UV	Ultraviolate
WPI	Whey protein isolate
WVTR	Water vapour transmission rate
WVP	Water vapour permeability
a_w	Water activity
D	Diffusion coefficient
E_a	Activation energy of diffusion
ΔH	Enthalpy of melting
k_1	Diffusion rate constant
k_2	Kinetic rate constant
l	Film thickness
m_t	Amount of AM agent released from the film
m_∞	Equilibrium amount of AM agent released from the film
R	Ideal gas constant
R^2	Correlation coefficient
T	Temperature
t	Time
T_m	Crystalline melting temperature
v_0	Initial rate of release of the AM agent
σ	Ultimate tensile strength
ε_b	Elongation at break
$E´$	Elastic modulus

1 Introduction

This chapter presents a background to microbial contamination of food products along with the microbial control on food products using various forms of active packaging (AP) technologies with an emphasis on antimicrobial (AM) packaging systems. This chapter also provides an insight into food packaging materials made from biobased polymers and in particular polysaccharides-based materials. The objectives, scope and thesis outline are also presented.

1.1 Background

1.1.1 Microbial Contamination of Food Products

Food products can be subjected to various types of contaminations including microbial contamination that is mainly caused by bacteria, yeasts and fungi (Ozdemir & Floros, 2004). Many of these microorganisms may cause undesirable reactions that deteriorate the flavour, odour, colour, sensory, and textual properties of foods (Appendini & Hotchkiss, 1997, 2002; Davidson & Taylor, 2007; Devlieghere *et al.*, 2004a; Gutierrez *et al.*, 2008; Han, 2005a; Rupika *et al.*, 2005; Vermeiren *et al.*, 1999; Weng *et al.*, 1999). Microbial growth in food products is one of the concerns from the perspective of spoilage and consumer safety because some microorganisms may potentially cause food-borne illness (Cha & Chinnan, 2004; Davidson *et al.*, 2005; De Olivera *et al.*, 2007; Natrajan & Sheldon, 2000b; Padgett *et al.*, 1998). In packaged foods, the growth and survival of common spoilage and pathogenic microorganisms such as *Listeria monocytogenes*, *Escherichia coli* O157, *Salmonella*, *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter*, *Clostridium perfringens*, *Aspergillus niger* and *Saccharomyce cerevisiae* is affected by a variety of intrinsic factors

such as pH, water activity, and the presence of oxygen or extrinsic factors associated with storage conditions including temperature, time and relative humidity (López-Malo *et al.*, 2005; Rydlo *et al.*, 2006; Singh *et al.*, 2003; Suppakul, 2004). Many food products including various types of cheeses, meats, poultry and baked products are highly susceptible to microbial spoilage (Limjaroen *et al.*, 2005; Schelz *et al.*, 2006; Silveira *et al.*, 2007; Weng & Hotchkiss, 1993). To prevent the growth of spoilage and pathogenic microorganisms on food products various traditional food preservation techniques such as heat treatment, salting, acidification and drying have been used in the food industry (Davidson & Taylor, 2007; Farkas, 2007; Ozdemir & Floros, 2004; Quintavalla & Vicini, 2002).

1.1.2 Microbial Control using Active Packaging Technologies

In recent years, a rise in consumers' demand for safe, fresh and minimally processed food products with an extended shelf-life has led to the increase in the development of new preservation techniques such as AP technologies that can provide safe food products with longer shelf lives (Devlieghere *et al.*, 2004a; Fitzgerald *et al.*, 2003; Han, 2005a; Lau & Wong, 2000; Ozdemir & Floros, 2004; Vermeiren *et al.*, 2002). Active packaging is a system in which the product, the package and the environment interact in a positive way to extend shelf-life or improve microbial safety or sensory properties while maintaining the quality of food products (Devlieghere *et al.*, 2000a; Han, 2000; Miltz *et al.*, 1995; Quintavalla & Vicini, 2002; Rooney, 1995; Suppakul *et al.*, 2003b). According to Rooney (1995) and Matche *et al.* (2004), the additional preservation roles rendered by AP systems to the packaged food product differentiates them from traditional packaging systems that offer only protective functions against external influences. Many researchers have published review articles in the area of active packaging systems with an emphasis on their potential food packaging applications (Appendini & Hotchkiss, 2002; Cooksey, 2001; Floros *et al.*, 1997; Han, 2000,

2003; Han, 2005a; Joerger, 2007; Kruijf *et al.*, 2002; López-Rubio *et al.*, 2004; Ozdemir & Floros, 2004; Quintavalla & Vicini, 2002; Suppakul *et al.*, 2003a; Vermeiren *et al.*, 2002).

1.2 Active Food Packaging

The AP technologies are designed primarily with the goal of protecting food products from deterioration and from the growth of microorganisms (Dainelli *et al.*, 2008; Juneja & Sofos, 2005). According to Miltz *et al*. (1995), Rooney (1995) and Smith (1995), many forms of AP technologies involve the use of films produced from polymeric materials. These materials can act as carriers (or matrices) for different active substances, including AM compounds, in order to maintain high concentrations of AM compounds on the food surfaces and/or to control and/or prevent the development and spread of spoilage and pathogenic microorganisms in food products (Krochta & De Mulder-Johnston, 1997; Raybaudi-Massilia *et al.*, 2008; Rojas-Graü *et al.*, 2009). Several researchers have defined AP technologies as those in which the internal atmosphere in the package is controlled by scavengers and emitters, moisture absorbers and preservative releasers (Matche *et al.*, 2004; Miltz *et al.*, 2006; Vermeiren *et al.*, 1999). The scavenging packaging systems are intended to remove undesirable compounds such as oxygen, carbon dioxide, ethylene, excessive water and taints from the packaged food product. Conversely, the emitters or preservative releasers add and/or emit compounds to the packaged food and/or into the head-space of the food package in order to inhibit the growth of microorganisms on the surfaces of the food (Anhvenainen, 2003). Common examples of AP systems include oxygen scavengers, carbon dioxide scavengers/emitters, moisture absorbers, ethylene scavengers/absorbers, ethanol emitters and AM systems (Appendini & Hotchkiss, 2002; Hotchkiss, 1997; Miltz *et al.*, 2006; Suppakul, 2004; Vermeiren *et al.*, 1999).

1.3 Active Scavenging and Emitting Packaging Systems

1.3.1 Oxygen Scavengers

Oxygen scavenging packaging represents an interesting phase in the development of active packaging systems in the food packaging industry. Such systems represent one of the AP technologies with many commercial successes particularly in Japan, Australia and the United States (Coma, 2008; Robertson, 2008). According to Solis and Rodgers (2001), oxygen scavenging packaging systems can be used in various packaging processes including compression moulding, casting, blown film extrusion and extrusion coating (Charles *et al.*, 2006). Oxygen in food packages originates from oxygen that enters through the packaging material, air encapsulated in the food, leakages due to poor sealing and/or poor gas flushing of the headspace (Juneja & Sofos, 2005; Ozdemir & Floros, 2004). The existence of oxygen in packaged food may initiate many food deterioration reactions (López-Rubio *et al.*, 2004). Oxygen may cause off-flavour (e.g. rancidity due to lipid oxidation), be detrimental to product's colour (e.g. pigment oxidation), cause nutrient losses (e.g. oxidation of vitamin C) and cause microbial growth (Miltz *et al.*, 2006; Suppakul *et al.*, 2003b; Vermeiren *et al.*, 1999). Though oxygen-sensitive foods can be packaged using high-oxygen barrier materials together with vacuum or modified atmosphere packaging, these techniques cannot completely remove oxygen inside the package (López-Rubio *et al.*, 2004). Therefore, such packaging techniques may be complemented with oxygen scavenger systems that reduce the residual oxygen remaining after packaging. The removal of residual oxygen is one of the effective methods for preventing growth of moulds and aerobic bacteria in dairy and bakery products (Smith *et al.*, 1995).

To reduce the rate of deterioration and spoilage reactions in food products, oxygen scavenging systems are based on the oxidation of one or more of these compounds: iron powder, ascorbic acid, photosensitive dyes, unsaturated fatty acids (e.g. oleic and linoleic acids) or enzymatic oxidation (e.g. glucose oxidase and alcohol oxidase) (Miltz *et al.*, 2006; Suppakul, 2004). According to Rooney (1995), the application of iron powder as an oxygen scavenging system was first developed and introduced to food packaging applications by Ageless® (Mitsubishi Gas Chemical Company, Japan). The iron powder is kept in sachets permeable to oxygen in which it is oxidised to iron oxide (Davidson *et al.*, 1993). According to Ozdemir and Floros, (2004), the amount of absorbent that should be in a sachet is determined by the amount of oxygen in the package, the permeability of the packaging material as well as the nature and the water activity of the food product. The oxygen scavenging systems using iron have the ability to absorb oxygen in both high and low moisture foods (Yam *et al.*, 2005). In many commercial applications, oxygen scavengers are included in sachets that are inserted into the package or bonded to the inner wall of the package (Han, 2000). Although this technology is well implemented, the oxygen scavenging sachets are not suitable for liquid foods because it is not desirable for the sachets to come in direct contact with liquid (Ozdemir & Floros, 2004). Furthermore, the sachets may be accidentally ingested with the food and/or impart an undesirable flavour or even be poisonous. The incorporation of scavengers into packaging films is one of the better ways of resolving the problems associated with sachets as the scavengers might be dissolved or dispersed in a polymer (Rooney, 1995). However, an alternative to dispersal of iron in a polymer is the utilization of organic reactions of polymers themselves such as the Oxyguard™ material (Vermeiren *et al.*, 1999).

1.3.2 Ethylene Scavengers

Another AP technology that has also been studied with relative commercial success is the ethylene scavenging system. Ethylene gas is a plant growth hormone that is produced by fruits and vegetables during ripening (Zagory, 1995). It accelerates ripening and senescence of fruits and vegetables (Miltz *et al.*, 2006). On the other hand, its accumulation can cause yellowing of green vegetables and may be responsible for a number of undesirable reactions, such as the development of bitter flavours, senescence and loss of chlorophyll (Zagory, 1995). Therefore, to extend the shelf-life and maintain an acceptable organoleptic quality, any accumulated ethylene inside the package should be removed (Brody & Bundy, 1995). The most common ethylene scavenger is potassium permanganate contained in sachets placed inside the package (Vermeiren *et al.*, 1999). Another ethylene scavenging system is based on impregnating zeolite with potassium permanganate and then coating the impregnated zeolite with a quaternary ammonium cation (Ozdemir & Floros, 2004). Ethylene scavengers have been shown to be effective in the storage of packaged fruits such as bananas and avocados. These are commercially available under different names, such as Evert-Fresh™ (Evert-Fresh Co., USA), Peakfresh™ (Peakfresh Products, Australia), Ethylene Control™ (Ethylene Control Incorporated, USA) and Orega™ plastic film (Cho Yang Heung San Co., Korea) (Zagory, 1995).

1.3.3 Carbon Dioxide Scavengers/Emitters

One of the successfully applied scavenging packaging technologies apart from oxygen and ethylene systems is the packaging system that functions by absorbing or emitting carbon dioxide into the head-space of packaged food products. Carbon dioxide emitting systems are incorporated with substances that release carbon dioxide into packaging matrices or sachets

in order to inhibit microbial growth in food packages (Ozdemir & Floros, 2004). In other cases, carbon dioxide concentration inside the package may increase in some products due to deterioration or respiration reactions (Robertson, 1993). The high concentration of carbon dioxide in a package exerts a microbiological inhibitory effect in products such as meat, cheese and baked goods (Suppakul *et al.*, 2003b). However, a high concentration of carbon dioxide may adversely affect the product due to anaerobic metabolism, pH reduction, off-colour and off-flavour development (López-Rubio *et al.*, 2004). Carbon dioxide is more permeable than oxygen through many films used for food packaging. If the packaging film has a high permeability to carbon dioxide, a carbon dioxide emitting system may be necessary to reduce the rate of respiration and suppress microbial growth (Robertson, 1993). Carbon dioxide emitting systems can be used in conjunction with oxygen scavenging systems in order to maintain an atmosphere favourable for microbial inhibition. Carbon dioxide emitting systems can prevent defects in package shape by maintaining relatively constant gas levels within packages (Suppakul *et al.*, 2003b). Commercial carbon dioxide scavengers make use of the sachet technology. The most common examples of carbon dioxide scavenging systems include calcium hydroxide (Vermeiren *et al.*, 1999) and zeolites (López-Rubio *et al.*, 2004).

In addition, inorganic gases such as sulfur dioxide (SO_2) and chlorine dioxide (ClO_2) have the potential of extending the shelf life of perishable foods by inhibiting the growth of spoilage microorganisms. These gases can be used to flush packages in order to prevent microbial growth, however incorporating them into sachets for controlled release into packages during storage may be more beneficial as far as AM packaging is concerned. Cooksey (2005) studied the application of ClO_2 emitting sachets, in combination with gas flushing, on the packaging of fresh chicken. Samples of chicken were packaged in barrier foam trays

containing either slow or fast release ClO_2 sachets and were flushed with either 100% N_2 or 75% N_2/25% CO_2 prior to storage at°C for 15 days. Samples treated with ClO_2 had significantly lower levels of *Salmonella typhimurium* during storage compared to the chicken stored without sachets, particularly those in a 75% N_2/25% CO_2 atmosphere.

1.4 Moisture Absorbers

The presence of excess water trapped in the packaged food during the packaging process is one of the major causes of food spoilage (López-Rubio *et al.*, 2004; Suppakul *et al.*, 2003a). According to López-Rubio *et al*. (2004), water-sensitive food products should be packaged with high moisture barrier materials. Some of the water can also be released during storage due to the respiration of fresh produce and/or temperature changes in a high humidity environment. If this water is not eliminated, it may appear as a condensate in the package (Suppakul *et al.*, 2003b). The water retained in the food product may facilitate the growth of microorganisms resulting in quality loss and shelf-life reductions, hence causing consumer rejection of the product (Vermeiren *et al.*, 1999).

The use of moisture-absorbing substances such as silica gel, calcium oxide, molecular sieves and calcium chloride is an effective way of controlling water inside a package (Rooney, 1995). Moisture-absorbing systems in sachet forms are used to maintain low levels of moisture in dried food packages (Smith *et al.*, 1995). The most widely used desiccant is silica gel due to its non-toxicity. A pad of desiccant is wrapped around the foods to be preserved in order to absorb water. The main purpose of the desiccant is to reduce water activity, inhibiting mould, yeast and/or bacterial growth. Sachets are also used to remove water released from frozen products such as

meat or fish and to prevent condensation of water in fresh products such as vegetables and fruits (Suppakul *et al.,* 2003a; Vermeiren *et al.,* 1999).

Another approach to absorb moisture from foods is to use a superabsorbent polymeric laminate film that has a moisture absorbent layer formed from a polyester graft copolymer and a resin, consisting of polyurethane and vinyl resin (Ozdemir & Floros, 2004). The commercial moisture absorbing systems used to absorb and/or control moisture in packaged foods include Thermarite®, Thermarite Pty. Ltd. Australia; Toppan Sheet™, Toppan Printing Co. Japan; Peaksorb®, Peakfresh Products Australia; Multisorb™, Multisorb Technologies, USA (López-Rubio *et al.*, 2004; Ozdemir & Floros, 2004; Vermeiren *et al.*, 1999). These materials are basically superabsorbent polymers placed between two layers of polyethylene or polypropylene. Moisture absorbers are used to reduce water activity, prevent oxidation and water condensation in the packaging of baked products, pasta and meat to inhibit the growth of bacteria, mould and yeasts (Appendini & Hotchkiss, 2002; Suppakul, 2004; Vermeiren *et al.*, 2002).

1.5 Ethanol Emitters

Ethanol has been found to be effective in extending the shelf life of food product by suppressing mould growth (Suppakul *et al.,* 2003b) and has been applied on surfaces, such as bread and other bakery products (Smith *et al.*, 1987). The application of ethanol as a microbial growth inhibitor in foods is usually achieved by the use of ethanol emitting sachets (Suppakul, 2004). The release of ethanol from the sachet into the package headspace is determined by the permeability of the sachet to water vapour (Appendini & Hotchkiss, 2002) because the ethanol in the sachet is exchanged with the water absorbed by the sachet. The effectiveness of an ethanol emitter depends on the type and size of the carrier material as well

as on the concentration of ethanol in it. The effectiveness also depends on the permeability of the sachet and/or packaging material to water vapour and ethanol, the water activity of the food, and the ethanol permeability of the packaging film (Robertson, 1993).

Films containing ethanol are not as widespread as sachets, due to the problems encountered in the controlled release of ethanol from the films into the package headspace (Smith *et al.*, 1995). Ethanol incorporated into films usually requires additional layers to hold the ethanol and to release it at a controlled rate (Appendini & Hotchkiss, 2002). Several advantages of ethanol-emitting systems include the direct emission of ethanol from ethanol-generating sachets in the package which stops the direct contact of ethanol with the food as outlined by Smith *et al*. (1987); hence such sachets provide safer foods compared to spraying of ethanol before packaging. In addition, ethanol-emitting sachets eliminate the need of other preservatives, such as sorbates and benzoates, for mould inhibition. However, the main disadvantage of ethanol-generating systems is the absorption of ethanol vapour by the food product from the package (Smith *et al.*, 1995). Smith *et al*. (1987) noted that when food is packed with a sachet of Ethicap™, moisture is absorbed from the food and encapsulated ethanol vapour is released from and permeates into the package headspace. Although the level of ethanol can be reduced to insignificant values by heating the product or exposing it to microwave radiation, food products consumed without being heated, may contain residual ethanol (Ozdemir & Floros, 2004).

1.6 Antimicrobial Packaging Systems

Antimicrobial packaging systems have attracted in recent years much attention in the food and packaging industries with the aim of replacing the conventional food preservation

systems that inhibit microbial spoilage (An *et al.*, 1998; Bagamboula *et al.*, 2004; Chung *et al.*, 2001a; Devlieghere *et al.*, 2004b; Miltz *et al.*, 2006; Quintavalla & Vicini, 2002; Weng & Hotchkiss, 1993). Different studies have shown that AM packaging systems can extend the shelf life of packaged foods by extending the lag phase and reducing the growth rate of spoilage microorganisms (Appendini & Hotchkiss, 2002; Coma, 2008; Cooksey, 2001; De Olivera *et al.*, 2007; Devlieghere *et al.*, 2004a; Han, 2000; Rardniyom *et al.*, 2008b; Rupika *et al.*, 2008b; Rydlo *et al.*, 2006; Suppakul *et al.*, 2011b). To diminish food spoilage by microorganisms, different AM agents are commonly incorporated directly into food products to preserve them from microbial contamination. This method has many disadvantages: (i) consumers today prefer foods with no or minimal synthetic additives because of concerns of side effects; (ii) since food spoilage occurs primarily on the surface, incorporation of relatively large quantities of the quite expensive agents in the bulk of the food is not justified; (iii) some of the synthetic agents possess a distinct flavour, rendering the flavour of the food, and (iv) synthetic additives have to be declared on the package which is a disadvantage from the consumers' attitude prospective. Antimicrobial agents that have the potential to be used in food packaging applications can be divided into synthetic and natural ones (Kuorwel *et al.*, 2011a).

Over the past few decades, various synthetic AM agents have been investigated and developed into food packaging materials (Weng & Hotchkiss, 1993; Weng & Hotchkiss, 1992). Many of these agents including various organic acids and salts have been approved by regulatory agencies and have since been used for the preservation of food products (Davidson & Taylor, 2007). Synthetic AM agents that have demonstrated inhibitory activity against different microorganisms include sodium benzoates and propionates, potassium sorbates, sulfites, chlorides, nitrites, triclosan, fungicides (e.g. benomyl, imazalil) and various metal

ions including silver zeolites, quaternary ammonium salts and copper ions (Chen *et al.*, 1996; Chung *et al.*, 2003a; Cooksey, 2005; Devlieghere *et al.*, 2000b; Han, 2000; Hoffman *et al.*, 2001; Ouattara *et al.*, 2000b). Other AM agents such as acetic acid from vinegar and benzoic acid from cranberries are found in nature, but are classified as synthetic agents when produced synthetically (Davidson & Taylor, 2007).

In recent years, natural AM agents have attracted much attention in the food and packaging industries as a replacement for synthetic ones for food preservation. According to Davidson and Zivanovic (2003), natural AM agents are classified by their sources: AM agents derived from plant EOs (e.g. basil, thyme, oregano, cinnamon, clove and rosemary); animal sources (e.g. lysozyme, lactoferrin); microbial sources (nisin, natamycin); and naturally occurring polymers (chitosan). The EOs extracted from plant sources consist of various mixtures including terpenoids, esters, aldehydes, ketones, acids and alcohols (Burt, 2004; Dorman & Deans, 2000; Roller, 2003). These plant EOs are volatile and generally possess relatively strong odours (Bakkali *et al.*, 2008).

Extracts derived from various herbs and EOs contain a range of natural compounds such as thymol, linalool and carvacrol which have a broad AM spectrum against different pathogenic and spoilage microorganisms, including gram negative species such as *E. coli, Yersinia enterocolitica, P. aeruginosa* and *Sal. choleraesuis* (Lim *et al.*, 2010b; Lopez *et al.*, 2007a; 2007b; Natrajan & Sheldon, 2000a; Suppakul *et al.*, 2011a), gram positive bacteria such as *L. monocytogenes, Staph. aureus, B.* cereus (Dawson *et al.*, 2002b; Friedman *et al.*, 2004a; Friedman *et al.*, 2002; Gutierrez *et al.*, 2009b; Lopez *et al.*, 2007a), yeasts such as *S. cerevisiae, Candida albicans, Debaryomyces hansenii* (Kuorwel *et al.*, 2011b; Rupika *et al.*, 2006; Suppakul *et al.*, 2008) and moulds such as *Alternaria alternate, A. niger, Botrytis*

cinerae, A. flavus, *penicllium roqueforti* (López-Malo *et al.*, 2007; Rodriguez-Lafuente *et al.*, 2010). These additives are considered to be safe and have the "Generally Recognised As Safe" (GRAS) status as designated by the American Food and Drug Administration–FDA (Han, 2005a; Matan *et al.*, 2006; Zaika, 1988).

Antimicrobial agents derived from plant sources are produced as secondary metabolites and are associated generally with the volatile EO fractions. The mode of action of AM agents and/or the AM activity of plant EOs is related to their chemical structure: namely, the presence of hydrophilic functional groups such as the hydroxyl groups in phenolic components and/or lipophilicity of the components in the EOs that depends on their concentration (Bagamboula *et al.*, 2004; Davidson & Naidu, 2000; Dorman & Deans, 2000; Farag *et al.*, 1989; Friedman *et al.*, 2002). Essential oils and their principal constituents inhibit microorganisms *via* a range of mechanisms such as: disruption of the cyctoplasmic membrane (Helander *et al.*, 1998; Knobloch *et al.*, 1989; Sikkema *et al.*, 1995); leakage of intracellular constituents such as metabolites and ions (Lambert *et al.*, 2001; Sikkema *et al.*, 1995); coagulation of cell content (Gustafson *et al.*, 1998; Pauli, 2001); inhibition of protein synthesis (Helander *et al.*, 1998), enzymes associated with cell wall synthesis (Conner & Beuchat, 1984), DNA/RNA synthesis (Tassou *et al.*, 2000; Ultee *et al.*, 1999), general/metabolite pathways (Ultee *et al.*, 2002); and/or the destruction of the osmotic integrity of the cell membrane (Ultee & Smid, 2001). The AM activity of different EOs is very difficult to compare given the variation of EOs compositions amongst the plant species, differences in their geographic origin, harvesting season, extraction methods and the part of plant that is used (Elgayyar *et al.*, 2001; Zaika, 1988).

1.7 Test Methods and Limitation of Antimicrobial Agents

There are a number of test methods used to determine the AM activity of various EOs and their principal constituents. These include the diffusion methods (agar diffusion), dilution methods (broth and agar dilution) and microatmosphere methods (Davidson & Zivanovic, 2003; Guynot *et al.*, 2003; Nedorostova *et al.*, 2011; Tunç & Duman, 2011). These methods provide preliminary information on the possible effectiveness of the tested active constituents (Kuorwel *et al.*, 2011b).

The agar diffusion method has been widely used in the past, but the results obtained from this technique are qualitative. Although the agar diffusion method can indicate the AM activity of EOs and/or their principal components on solid media, the high hydrophobicity of EOs is always a major problem (Davidson & Zivanovic, 2003; Friedman *et al.*, 2002). As a result, the agar disc diffusion assays do not generally demonstrate a clear zone of inhibition at very low AM agent concentrations; however these do exhibit a clear inhibition zone at high concentrations of hydrophobic, lipophilic AM agents (Sanla-Ead *et al.*, 2011). On the other hand, microatmosphere methods, which allow the determination of the AM activity of EOs and/or their constituents in the vapour phase, can be used with lipophilic AM films at low concentrations of AM agents (Fisher *et al.*, 2009; Goñi *et al.*, 2009; Kloucek *et al.*, 2011; Lopez *et al.*, 2007a; Suhr & Nielsen, 2003). Recently, Sanla-Ead *et al.* (2011) investigated the AM activity of cinnamaldehyde and eugenol incorporated into cellulose-based packaging films against Gram-negative bacteria (*E. coli*, *Sal. enteritidis*), Gram-positive bacteria (*L. monocytogenes*, *Staph. aureus*) and yeasts (*C. albicans*, *C. cerevisiae)* using the vapour diffusion assay. The authors reported that cinnamaldehyde and eugenol incorporated into cellulose-based packaging films demonstrated positive inhibitory effects against the tested microorganisms.

1.8 Limitation of Antimicrobial Agents

The AM activity of many EOs and their active components can be affected by factors such as solubility of AM agents in food systems or microbiological media, volatility and lipophility of the AM agents in the food systems (Davidson & Naidu, 2000; Friedman *et al.*, 2002; Lambert & Pearson, 2000). These factors can cause significant variation of results of AM activity for various studies utilising the same AM agents unless the factors are controlled.

1.8.1 Solubility of Antimicrobial Agents

Solubility of EOs and their non-polar active components is one of the problems that can affect their AM efficacy. It is generally accepted that EOs are more effective in microbiological media than in food systems. This phenomenon can be explained in terms of solubility and phase distribution parameters. The limited solubility of essential oils and many of their constituents in aqueous media is likely to impair their performance in susceptibility tests. Attempts have been made to overcome this problem by using surfactants such as Tween™-20 and Tween™-80 (Paster *et al.*, 1990). However, in many cases, surfactants counteract the action of these AM agents (Manou *et al.*, 1998; Paster *et al.*, 1990). Paster *et al.*, (1990) observed a reduction in the AM activity of thymol with increasing concentration of Tween™-80, and assumed that this surfactant increases the solubility of thymol in water and reduces the solubility in the bacterial cell membrane. Tween™-80 may cause thymol and membrane proteins to become more hydrophilic and thus reduce the degree of binding between them. The presence of agar as a stabiliser in broth substantially improves the AM properties of OEO and TEO. This is assumed to be attributable to the reduced separation of the EO from the water phase, which would enable more effective inhibition of bacterial cells (Burt & Reinders, 2003).

1.8.2 The volatility and lipophilicity of Antimicrobial Agents

The limitations of the AM agents derived from EOs is their volatility and lipophilicity in food systems (Bagamboula *et al.*, 2004). In the food matrix, the AM activity of EOs and their constituents decreases due to the interactions between phenolic compounds and the components in the food matrix such as proteins, fats, sugars, salts (Nevas *et al.*, 2004; Nychas & Tassou, 2000; Vigil, 2005). Some of the constituents of EOs can partition into the lipid phase due to their lipophilicity thereby causing some components to lose their AM activity. Therefore, only a small amount of the AM agent added to the food matrix may retain its AM activity (Nychas & Tassou, 2000). Several studies have demonstrated that the concentration and type of oil or fat present in a food can affect the AM efficacy of EOs or their components (Mejlholm & Dalgaard, 2002) and (Smith-Palmer *et al.*, 2001). In their study, Mejlholm and Dalgaard (2002) have claimed that partitioning of the hydrophobic antibacterial EO components into the fat content of the food may prevent them from coming in contact with the target bacterial cells growing in the hydrophilic or aqueous regions. Nevertheless, some constituents of thyme and oregano EOs such as thymol and carvacrol are phenolic compounds that can benefit from lipophilicity. Due to their phenolic nature, the mode of AM activity of thymol and carvacrol is considered to be on the cell membranes that can benefit from lipophilicity due to their affinity to the hydrophobic domain of the cytoplasmic membrane of bacterial cells (Manou *et al.*, 1998).

1.9 Biobased Packaging Materials

Consumer demands and requirements by regulatory agencies to use more environmentally-friendly and less polluting packages have directed researchers to consider packaging materials that are derived from natural or made from renewable resources to replace, at least

some of the synthetic polymers (Khwaldia *et al.*, 2010; López-Rubio *et al.*, 2004; Ponce *et al.*, 2008; Weber *et al.*, 2002). Biobased polymers can be produced from natural, renewable resources (e.g. starch), chemically synthesised from natural sources (e.g. poly(lactic acid)) or made from microbiologically produced materials (e.g. hydroxybutyrate and hydroxyvalerate) (Cagri *et al.*, 2004; Cha & Chinnan, 2004; Perez-Gago & Krochta, 2005; Petersen *et al.*, 1999; Pommet *et al.*, 2005; Weber *et al.*, 2002). These biopolymers have the potential to be substitutes for conventional packaging materials that are derived from crude oils (Bertuzzi *et al.*, 2007). Although all the biobased materials are not necessarily biodegradable (Marsh & Bugusu, 2007), some of the biopolymers such those obtained from agricultural sources can decompose more readily in the environment than their synthetic polymeric counterparts that are derived from crude oils (Altskär *et al.*, 2008; Chick & Ustunol, 1998; Cutter, 2006; Dias *et al.*, 2010; Iovino *et al.*, 2008; Lopez-Rubio *et al.*, 2006; Tharanathan, 2003). It is important to note that not all biobased materials are degradable in a land fill since chemical modification of some biobased polymers can have significant effect on their rate of degradation (Nair & Laurencin, 2007). According to Marsh & Bugusu (2007), a change from synthetic polymers to biopolymers might have little impact on source reduction and incineration. Recycling could be complicated by the presence of blended or modified polymers if not separated from the recycling stream.

Biobased materials derived from polysaccharides and proteins, when combined with AM agents, have the potential to be manufactured into food packaging films with effective AM properties (Cha & Chinnan, 2004; Dawson *et al.*, 2002a; Hernandez-Izquierdo *et al.*, 2008a; Krochta & De Mulder-Johnston, 1997; Marcos *et al.*, 2010; Ozdemir & Floros, 2001; Petersen *et al.*, 1999). Polysaccharide-based materials with AM agents, particularly the starch-based ones, have been studied extensively with some commercial success in the food

packaging industry (De Vlieger, 2003; García *et al.*, 2009; Guilbert & Gontard, 2005; Robertson, 2008). Some modified biobased polymers such as starch-based materials can be manufactured into films and used to package dry and/or solid food products such as biscuits, snacks, cereals, fresh produce, fruits and vegetables (Gennadios *et al.*, 1997; Krochta & De Mulder-Johnston, 1997; Lacroix & Le Tien, 2005; Nisperos-Carriedo, 1994). Recently, commercially developed starch-based packaging materials have become available including Plantic®, EverCorn™ and Bio-P™ made by Plantic Technologies, Novamont and Bioenvelope, respectively (García *et al.*, 2009; Robertson, 2008).

Developing commercial biobased films with improved physical and mechanical properties is still a challenge due to their hydrophilic nature that limits their application for packaging of food products with a high water activity (Gennadios *et al.*, 1997; Lacroix & Le Tien, 2005; Olivas & Barbosa-Cánovas, 2008; Robertson, 2007b; Wong *et al.*, 1994). Antimicrobial packaging films with improved physical and mechanical properties could be prepared from biobased polymers that have been modified and/or blended with other compatible materials incorporated or coated with AM agents (Flores *et al.*, 2007b; Imran & Erbatur, 2008; Rhim & Shellhammer, 2005). However, additional research and development work is required to reduce the moisture sensitivity of these polymers, enhance their physical properties and improve their processability (Guilbert & Gontard, 2005). These goals can be achieved by proper blending with appropriate materials and/or by copolymerisation (Arvanitoyannis & Biliaderis, 1999; Davis & Song, 2006; Garcia *et al.*, 2000a; Marron *et al.*, 2000; Tharanathan, 2003; Zhang *et al.*, 2009). Biobased materials could also be successfully prepared and applied in AM packaging systems by the incorporation of appropriate AM agents (Bastarrachea *et al.*, 2010; Han, 2005b; Kuorwel *et al.*, 2011b; Pranoto *et al.*, 2005a). Taking into consideration that the public, as a whole, is already conscious (and becomes even more so as times go by)

to the environment, it is conceivable that the future will see more biobased and AM biobased polymers and/or their devivatives in the packaging of food, agricultural and other products (Guilbert & Gontard, 2005; Han, 2005b; Rhim & Shellhammer, 2005). Although there is an increasing concern in recent years about the recycling of synthetic polymers due to their non-composition, synthetic polymers used in food packaging have still many advantages such as low cost, good process-ability and good mechanical and physical properties (Guilbert & Gontard, 2005). Therefore, the development of AM packaging materials manufactured from synthetic polymers such as low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyethylene-terephthalate (PET), ethylene vinyl acetate (EVA), polystyrene (PS) and polypropylene (PP) has the potential to deliver commercial benefits for the packaging of various food products (Iconomopoulou & Voyiatzis, 2005; Lee *et al.*, 2008; Nostro *et al.*, 2010; Pawde & Sanmesh, 2008; Rupika *et al.*, 2008b).

1.10 Aims

The main objective of the present research was to prepare viable AM films from starch-based substrate materials by heat pressing and/or coating processes and to incorporate natural AM agents: linalool, carvacrol and thymol at various concentrations in their structure. In particular, the research focused on the effects of AM agents on the food-borne pathogens *Staphylococcus aureus, Saccharomyce cerevisiae* and *Aspergillus niger* as these are of concern in the packaging of Cheddar cheese which is seen as a possible commercial application for the outcomes of the project. The following were the specific objectives of this project:

- To investigate the water uptake and the mechanical properties of starch-based films as a function of water content and/or RH in order to identify the maximum level at which the films can be used practicably as a packaging medium;
- To successfully prepare AM films by heat pressing and/or coating of the starch-based films with AM agents, in view of their potential for food packaging applications;
- To determine the residual concentrations of AM agents retained in the films after heat pressing and/or coatings process;
- To investigate the migration of linalool, carvacrol or thymol incorporated into or coated onto starch-based films into the fatty food simulant, isooctane, recommended by the US Food and Drug Administration (FDA, 2007) in order to mimic the packaging environment of a fatty product like Cheddar cheese;
- To investigate the temperature dependency of the AM agents migration into isooctane;
- To investigate the loss to the atmosphere of AM agents from these packaging systems and of their AM retention during storage;
- To evaluate the effectiveness of starch-based films coated with linalool, carvacrol or thymol against *S. aureus*, *S. cerevisae* and *A. niger in vitro* or inoculated on the surfaces of Cheddar cheese samples;
- To evaluate the AM activity at several concentrations of each of the AM agents coated onto a starch-based polymer film and to determine the relative order of sensitivity of microorganisms to each AM agent.

1.11 The Scope of Work

It has been demonstrated in previous studies that linalool, carvacrol, and thymol (the constituents of basil, oregano and thyme essential oils respectively) have potential AM activities in food packaging applications. However, most studies have focused mainly on the characterisation and development of systems using synthetic polymers such as LDPE and testing their efficacy in model systems and not in biobased polymers. In the present study, the inhibitory effects of AM agents linalool, carvacrol or thymol have been incorporated into starch-based materials. The AM starch-based films so produced were then tested on solid media as well as on Cheddar cheese. The post-processing retention of AM agent in the films was determined and the loss of AM agent from stored film was also monitored. Furthermore, the suitability of starch-based materials to be used for food packaging applications has been explored and assessed.

1.12 Thesis Outline

This thesis contains five chapters, a reference list and three appendices. Chapter 1 is an introductory chapter that leads to the aims and includes the scope of the thesis. Chapter 2 presents the literature review with much emphasis on active packaging systems utilising biobased packaging materials as well as the factors that affect the properties and performance of the biobased films. The AM activity of natural AM agents derived from volatile EOs is also discussed. The materials and methods for this study are presented in Chapter 3. The experimental procedure is subdivided into three sections which include film preparation and characterisation, *in vitro* evaluation of the AM films and the application of AM films on Cheddar cheese. The results and discussion of this study in relation to other studies reported in the literature are presented in Chapter 4. In Chapter 5, the conclusions and the significance

of results in relation to the aims of the project are discussed. Furthermore, recommendations for further investigations are also presented in the final chapter. Appendix A covers the properties of the AM agents, microorganisms and polymers used in this study. Some of the supplementary figures pertaining properties of starch-based films are presented in Appendix B. Appendix C covers supplementary figures for the migration of AM agents into food simulants. Finally, appendix D covers supplementary figures for the AM of AM films against reference microorganisms on solid media.

2 Literature Review

In the present chapter, the concept of AM packaging systems with respect to food packaging applications is considered with a focus on biobased films, mainly polysaccharides and protein-based materials. This is followed by a detailed discussion about various forms of films incorporated and/or coated with AM agents. This chapter also provides a detailed overview of each of the factors that may affect the performance characteristics of biopolymers used as food packaging materials with an emphasis on AM agents and plasticisers. Finally, a detailed summary of synthetic films utilising common synthetic and natural AM agents is presented with an emphasis on the principal components of basil, oregano, and thyme essential oils namely, linalool, carvacrol and thymol respectively.

2.1 Polysaccharides and Proteins-Based Materials

In recent years interest has increased on the potential uses of films and coatings manufactured from biobased polymers particularly polysaccharides and protein-based materials. In the last two decades the interest in these materials has been primarily for their possible use in food packaging (Baldwin *et al.*, 1995; Krochta *et al.*, 1994; Krochta & De Mulder-Johnston, 1997). Polysaccharides and proteins-based films demonstrate adequate gas barrier properties (Hernandez-Izquierdo & Krochta, 2008b). Examples of polysaccharide-based polymers that have a potential to be used in antimicrobial (AM) packaging systems or may be used in conjunction with AM agents include starch, alginate, cellulose, chitosan, carageenan. Examples of proteins-based materials include whey protein, soya protein, corn zein and/or their derivatives (Brody, 2005; Cagri *et al.*, 2004; Dawson *et al.*, 2002a; Krochta, 2002; Min *et al.*, 2005a; 2005b; Phan *et al.*, 2005; Rodriguez *et al.*, 2006; Shellhammer & Krochta,

1997). Furthermore, various forms of polysaccharides, protein-based polymers and/or other biobased polymers identified by Weber (2002) have the potential to be developed into active packaging materials for food packaging applications. Many biobased materials such as polysaccharides and protein-based polymers are hydrophilic with a relatively high degree of crystallinity causing processing and performance difficulties. Thus, AM packages made from such biobased films demonstrate high moisture sensitivity, poor water barrier and poor mechanical properties compared to those made from synthetic polymers (Weber *et al.*, 2002).

Packaging materials with suitable physico-mechanical properties can nonetheless be prepared from biopolymers such as starch-based materials when the biobased materials are modified by physical, mechanical and/or chemical techniques or by blending them with compatible plasticisers (Arvanitoyannis & Biliaderis, 1998a; Davis & Song, 2006; Fang *et al.*, 2005; Garcia *et al.*, 2000b; Pommet *et al.*, 2005; Tharanathan, 2003; 1999). Plasticisers are relatively low molecular weight compounds that can be copolymerised with the polymer or added to the polymer in order to reduce the intermolecular and intramolecular forces and thereby increase the mobility of the polymeric chains (Garcia *et al.*, 2000a; Sothornvit & Krochta, 2005; Tharanathan, 2003). Plasticisers are usually mixed with biopolymers to improve processing, increase film flexibility and lower the glass transition temperature (Arvanitoyannis & Biliaderis, 1999; Avérous *et al.*, 2000; Brody, 2005; Fang *et al.*, 2005; Krochta, 2002; López *et al.*, 2008; Zhang & Liu, 2009). Examples of plasticisers that are commonly used with biopolymers include polyols such as glycerol, sorbitol and mannitol, monosaccharides such as fructose, glucose and mannose, and poly(ethylene glycol) (Brody, 2005; Kester & Fennema, 1986). Water is another important plasticiser for biobased films although excess moisture may affect the film properties (Krochta, 2002; Van Soest & Essers, 1997). Water can be added to a starch-based film in order to break its native granular

structure and hydrogen bonding (Mali *et al.*, 2002; Myllärinen *et al.*, 2002b; Yang & Paulson, 2000).

When a biopolymer is chemically, mechanically or physically modified, it is capable of exhibiting thermoplastic properties (Arvanitoyannis & Biliaderis, 1999). Modified biobased materials such as starch can thus be manufactured into a suitable packaging film using conventional plastic conversion processes like compression moulding, extrusion and thermoforming (Carvalho *et al.*, 2005; Jin & Zhang, 2008; Kim *et al.*, 2002b; Kristo *et al.*, 2008). Packaging films made from biobased polymers such as polysaccharides exhibit low gas permeability, enabling the extension of shelf life of food products without creating anaerobic conditions (Baldwin *et al.*, 1995). These biobased films or coatings can also be used to prolong the shelf-life of foods such as muscle food products by preventing dehydration, oxidative rancidity and surface browning (Nisperos-Carriedo, 1994). In the last decade, commercially developed starch-based packaging materials like Plantic®, EverCorn™ and Bio-P™ made by Plantic Technologies, Novamont and Bioenvelope respectively, became available (García *et al.*, 2009; Robertson, 2008). These materials can be used in commercial applications to package food products such as biscuits and snacks. Biobased materials have also found successful applications in the pharmaceutical industry as films or coatings to control drug release (Arifin *et al.*, 2006; Siepmann *et al.*, 2004; Soppimath *et al.*, 2001; Tuovinen *et al.*, 2003).

2.2 Preparation of AM Films from Biobased Materials

The main processing techniques used for the preparation of biobased films include wet and dry processing methods (Brody, 2005; Pommet *et al.*, 2005). The wet methods comprise

solvent casting (which is the most commonly used laboratory-scale technique to prepare AM films from biopolymers) whereas the dry methods involve usually compression moulding or extrusion of the biopolymers that have been modified to become thermoplastic (Chaléat *et al.*, 2008; 2006; Liu *et al.*, 2006; Mehyar & Han, 2004; Nam *et al.*, 2007; Pommet *et al.*, 2005; Thunwall *et al.*, 2006a; Van Soest & Essers, 1997). The processing techniques may significantly affect the properties of the resultant AM film made from a biobased material (Altskär *et al.*, 2008). Different factors affect the choice of the processing techniques when preparing an AM packaging film (Han, 2005a). These include the type and properties of the polymer, the properties of the AM agent (such as polarity and compatibility with the polymer), the heat stability of the latter during processing and the residual AM activity after manufacturing (Han, 2000). When a polar AM agent is added to a non-polar polymer to produce an AM film, the incorporated AM agent may affect the physical and mechanical properties of the resultant AM film (Han, 2003). However, if the AM agent is compatible with the polymer, a considerable amount of it can be incorporated into the packaging material with minimal physico-mechanical property deterioration (Han, 2005a; Han & Floros, 1997; Rupika, 2008a; Suppakul, 2004). Therefore, the polymer and/or the AM agent may require modification prior to film processing in order to increase the compatibility between the two (Cha & Chinnan, 2004). During manufacturing of AM films, the temperature and the shearing forces must be carefully considered (Han, 2003). High processing temperatures may result in considerable losses of volatile AM agents (Han, 2000; Han & Floros, 1997; Rupika *et al.*, 2005). Cooksey (2005) suggested that the AM agent might partly or completely lose its AM activity when incorporated into the film under harsh processing conditions. Nam *et al.* (2007) reported up to 48% recovery of the initial lysozyme activity in an extruded starch-based film upon increasing the extrusion temperature. Therefore, to minimise the loss of AM

agent during processing, as low as possible temperatures should be applied as recommended by Han and Floros (1998a).

2.3 The Antimicrobial Activity of Biobased Films

Numerous studies have identified migratory and non-migratory systems as the two main types of AM packaging systems. A migratory system contains an AM agent that can migrate into the headspace of the package. A non-migratory system contains an AM agent immobilised onto the packaging film. In the latter case, the AM film becomes effective against microbial growth when the food and the packaging material are in direct contact (Appendini & Hotchkiss, 1997, 2002; Brody *et al.*, 2001; Davidson *et al.*, 2005; Han, 2005b; Vermeiren *et al.*, 2002). These forms of AM packaging systems are designed primarily for the purpose of protecting food products from deterioration and spoilage by microorganisms. The following subsections provide a detailed overview of each of the different forms of AM packaging systems by utilising biobased films. Table 2.1 shows that significant progress has been made by effectively integrating AM agents into various biobased polymers, particularly polysaccharides such as starch-based and protein-based films. Such AM films have demonstrated inhibitory activity against the growth of various microorganisms. Understandably, the physico-mechanical properties of the films are other important aspects to be considered when designing the film for food packaging applications.

2.4 The AM Activity of Biobased Films Containing AM Agents

Impregnation of an AM agent into a packaging material is a feasible means for achieving good AM activity of an AM film (Han, 2003; Suppakul *et al.*, 2003a; Weng & Hotchkiss, 1993). This method enables a slow release of the agent onto the food surfaces and to maintain

an adequate concentration of the agent to effectively inhibit microbial growth throughout the product shelf life (Cooksey, 2005; Salleh *et al.*, 2007). An AM agent can be incorporated into a packaging material by blending it with a base polymer before manufacturing (extrusion or compression moulding) of the film (Mistry, 2006; Rardniyom *et al.*, 2008b; Rupika *et al.*, 2008b; Suppakul *et al.*, 2006). This method enables the AM agent to be evenly distributed in the amorphous region of the material (Suppakul, 2004).

Table 2.1 Antimicrobial activity of AM agents in biobased materials

Packaging Material	Antimicrobial Agent	Loading	Application[a]	Substrate	Microorganism(s)	Observations	References
Polysaccharide Films							
Calcium alginate	Acetic acid	2% (v/v)	C	Lean beef tissue	*L. monocytogenes*	Reduced *L. monocytogenes* growth	Siragusa and Dickson (1992)
Calcium alginate	Acetic acid	2% (v/v)	C	Lean beef tissue	*L. monocytogenes*, *S. typhimurium* and *E. coli O157:H7*	Decreased *L. monocytogenes*, *S. Typhimurium*, *E. coli O157:H7*	Siragusa and Dickson (1993)
Calcium alginate	Lactic acid	1.7% (v/v)	IM	Lean beef tissue	*L. monocytogenes*	Reduced *L. monocytogenes* count	Siragusa and Dickson (1992)
Calcium alginate gel	Nisin	1×10^2 µg/mL	IM	Lean and adipose beef carcass	*B. thermosphacta*	Reduced 2.84 and 2.91 log of *B. thermosphacta* on lean and adipose respectively	Cutter and Siragusa (1996; 1997)
Cellulose casing	Pediocin	10% (w/v)	C	Fresh poultry, fresh beef, ham	*L. monocytogenes*	Inhibited growth of *L. monocytogenes* in fresh and processed products	Ming *et al.* (1997)
Cellulose	Nisin		IN	Agar medium	*L. innocua* and *S. aureus*	Inhibited growth of *L. innocua* and *S. aureus*	Coma *et al.* (2001)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
Cellulose film	Olive leaf extract	0.5-3% (w/v)	IN	Agar method Cheese	*S. aureus*	Decrease 1.22 log of *S. aureus* after 14 days	Ayana and Nazan (2009)
Cellulose, chitosan	Potassium sorbate	2-5% (w/v)	C	Agar diffusion	*Rhodotorula rubra* and *Penicillium notatum*	AM activity against *R. rubra* and *P. notatum*	Chen *et al.* (1996)
Cellulose, chitosan	Sodium benzoate	2-5% (w/v)	C	Agar diffusion	*Rhodotorula rubra* and *Penicillium notatum*	AM activity against *R. rubra* and *P. notatum*	Chen *et al.* (1996)
Chitosan	Nisin	4.63-37.04 × 10^2 IU	IN	Agar diffusion	*S. aureus*, *L. monocytogenes*, *B. cereus*, and *E. coli*	Inhibited growth of *S. aureus*, *L. monocytogenes* and *B. cereus* but not *E. coli*	Li *et al.* (2006)
Chitosan	Acetic acid	1% (w/v)	IN	Ham, bologna, pastrami	*S. liquefaciens*, and *L. sakei*	Reduced growth of *S. liquefaciens* and *L. sakei*	Ouattara *et al.* (2000a)
Chitosan	Acetic acid	0.25-1% (w/v)	IN	Ham, bologna, pastrami	*Enterobacteriaceae*, *S. liquefaciens*, *L. sakei*	Growth of *S. liquefaciens* was delayed by film	Ouattara *et al.* (2000a)
Chitosan	Cinnamon oil	0.4-2% (v/v)	IN	Agar method	*L. monocytogenes*, *L.*	Iinhibited *L. monocytogenes*, *L. plantarum*, *E. coli*, *L. sakei* , *P.*	Ojagh *et al.* (2010b)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
					plantarum, E. coli ,L. sakei, Ps. fluorescens	*fluorescens*	
Chitosan	Garlic oil	1-4 × 10^2 µg/g	IN	Agar method	*E. coli, S. aureus, S. typhimurium, L. monocytogenes* and *B. cereus*	Clear zone of inhibition against *S. aureus, L. monocytogenes* and *B. cereus*	Pranoto *et al.* (2005b)
Chitosan	Nisin	5.1-204 × 10^3 IU/g chitosan	IN	Agar method	*E. coli, S. aureus, S. typhimurium, L. monocytogenes* and *B. cereus*	Film inhibited growth of S. *aureus, L. monocytogenes* and *B. cereus*	Pranoto *et al.* (2005b)
Chitosan	Potassium sorbate	50-200 mg/g	IN	Agar method	*E. coli, S. aureus, S. typhimurium, L. monocytogenes* and *B. cereus*	Demonstrated AM activity against *S. aureus, L. monocytogenes* and *B. cereus*	Pranoto *et al.* (2005b)
Chitosan	Propionic acid	1% (w/v)	IN	Ham, bologna, pastrami	*S. liquefaciens, L. sakei*	All films reduced growth of *S. liquefacien*s for all the storage	Ouattara *et al.* (2000b)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
						period.	
Chitosan	Lysozyme	60% (w/w)		Agar media	*E. coli* and *L. monocytogenes*	AM activity against E. *coli* and *L. monocytogenes*	Duan *et al.* (2008)
Chitosan-HPMC	Chitosan	0.5-2% (w/v)		Agar method	*L. monocytogenes*	Inhibited *L. monocytogenes*	Möller *et al.* (2004)
PLA	Nisin	0.25 g/mL	IN	Liquid culture, orange juice, egg white	*E. coli O157:H7, S. enteritidis*, and *L. monocytogenes*	Films reduced growth of *E. coli O157:H7*, *S. enteritidis*, and *L. monocytogenes*	Jin and Zhang (2008)
Starch-based	Dermaseptin S4	3 mg/L	C	Cucumber	Moulds and aerobic bacteria	Film demonstrated AM activity	Miltz *et al.* (2006)
Starch	Grape seed extract	1-20% (w/v)	IN	Agar media Pork loin	*L. monocytogenes*, *E. coli*, *E. faecalis*, , *E. faecium*, *S. typhimurium*, and *B. thermosphaceta B2*	Reduced growth of *thermosphaceta B2* on pork loin; inhibited Gram-positive bacteria on solid media but not Gram-negative bacteria	Corrales *et al.* (2009)
Starch film	Chitosan	1-9% (w/w)	IN	agar and liquid media	*B. subtilis* and *E. coli*	Inhibited *B. subtilis* and *E. coli*	Salleh *et al.* (2007)

Packaging Material	Antimicrobial Agent	Loading	Application[a]	Substrate	Microorganism(s)	Observations	References
Starch film	Chitosan	5-15% (w/w)	IN	Agar media and semisolid	*E. coli* and *S. aureus*	Inhibited both *E. coli* and *S. aureus*	Shen *et al.* (2010)
Starch film	Chitosan	1-5% (w/v)	IN	Liquid culture	*S. enteritidis*	Inhibitory effect against *S. enteritidis*	Durango *et al.* (2006)
Starch film	Lauric acid	8% (w/w)	IN	Agar and liquid culture media	*B. subtilis* and *E. coli*	Inhibition of *B. subtilis* and *E. coli*	Salleh *et al.* (2007)
Starch film	Lysozyme	1% (w/w)	IN	Agar media	*B. thermosphaceta B2*	Inhibitory effect against *B. thermosphaceta B2*	Nam *et al.* (2007)
Starch film	Potassium sorbate	5-15% (w/w)	IN	Agar media semisolid	*E. coli* and *S. aureus*	Inhibited *E. coli* but not *S. aureus*	Shen *et al.* (2010)
Starch film	Potassium sorbate	20%	IN	Liquid culture, poultry	*S. typhimurium* and *E. coli*	Inhibited *S. typhimurium* and *E. coli O157:H7* by 4 and 2 logs respectively	Baron and Sumner (1993)
Starch-alginate	Lemongrass oil	0.1-0.4% (w/v)	IN	Agar media	*E. coli O157:H7*	Inhibited *E. coli O157:H7* growth	Maizura *et al.* (2008)
Starch-chitosan	Oregano EOs	0.1-1% (w/w)	IN	Agar media	*E. coli O157:H7, S. aureus, S. enteriditis*, and	Inhibited *E. coli O157:H7, S. aureus, S. enteriditis, B. cereus*	Pelissari *et al.* (2009)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
					B. cereus		
Starch	Grape seed extract	1-20% (w/v)	IN	Agar media Pork loin	*L. monocytogenes*, *E. coli*, *E. faecalis*, *E. faecium*, *S. typhimurium*, and *B. thermosphaceta B2*	Reduced 1.3 log CFU mL^{-1} of *B. thermosphaceta B2* on pork loin; inhibited Gram-positive bacteria on solid media but not Gram-negative bacteria	Corrales *et al.* (2009)
Protein Films							
Corn zein	Calcium-propionate	1% (w/w)	C	Ready-to-eat chicken	*L. monocytogenes*	Coated films suppressed *L. monocytogenes* growth	Janes *et al.* (2002)
Corn zein	Lysozyme	479-958 µg/cm^2	IN	Agar media	*E. coli* and *B. subtilis*	Effective against *E. coli* and *B. substilis*	Güçbilmez *et al.* (2007)
Corn zein	Nisin	1× 10^3 IU/g	C	Ready-to-eat chicken	*L. monocytogenes*	Coated films reduced *L. monocytogenes* growth	Janes *et al.* (2002)
Corn zein	Lauric acid	200 mg	IN	Liquid culture	*L. monocytogenes*, and *S. enteriditis*	Significant effect against *L. monocytogenes* but not against *S. enteriditis*	Hoffman *et al.* (2001)
Corn zein	Nisin	0.188 mg	IN	Liquid culture	*L. monocytogenes*, and *S.*	Reduced counts of *L. monocytogenes*, *S. enteriditis*	Hoffman *et al.* (2001)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
					enteriditis		
Proteins-based film	Oregano Eos	1% (w/v)	IN	Beef muscle slices	*Pseudomonas spp.* and *E. coli H0157:H7*	Films containing oregano reduced 0.95 and 1.12 log of *P. spp.* and *E. coli H0157:H7* respectively, after 7 days	Oussalah *et al.* (2004)
Proteins-based film	Pimento Eos	1% (w/v)	IN	Beef muscle slices	*Pseudomonas spp.* and *E. coli H0157:H7*	Films containing pimento EOs were reported to be less effective against *E. coli H0157:H7* and *Pseudomonas*	Oussalah *et al.* (2004)
Sodium caseinate	Nisin	7.5-75 × 10^{-4} (w/w)	IN	Agar media	*L. monocytogenes*	Effectively reduced *L. monocytogenes*	Kristo *et al.* (2008)
Sodium caseinate	Potassium sorbate	10-25 (w/w)	IN	Agar media	*L. monocytogenes*	Reduced growth of *L. monocytogenes*	Kristo *et al.* (2008)
Sodium caseinate	Sodium lactate	10-40 (w/w)	IN	Agar media	*L. monocytogenes*	Slightly effective against *L. monocytogenes*	Kristo *et al.* (2008)
Soy protein Corn zein	EDTA	15-30m mM	IN	Agar and liquid media	*L. plantarum* and *E. coli*	Inhibited *E. coli* at 30 mM	Padgett *et al.* (1998; 2000)

Packaging Material	Antimicrobial Agent	Loading	Application[a]	Substrate	Microorganism(s)	Observations	References
Soy protein Corn zein	Lauric acid	2.5-133 mg/g	IN	Agar and liquid media	*L. plantarum* and *E. coli*	Inhibited *L. plantarum* but not *E. coli*	Padgett *et al.* (1998; 2000)
Soy protein Corn zein	Lysozyme	2.5-133 mg/g of film	IN	Agar and liquid media	*L. plantarum* and *E. coli*	Inhibited *L. plantarum* and *E. coli*	Padgett *et al.* (1998; 2000)
Soy protein Corn zein	Nisin	0.01-6 mg/g of film	IN	Agar and liquid media	*L. plantarum* and *E. coli*	Inhibited *L. plantarum* and *E. coli.*	Padgett *et al.* (1998; 2000)
Soy protein isolate	EDTA	0.16% (w/w)	IN	Liquid or solid media	*E. coli O157:H7, S. typhimurium*, and *L. monocytogenes*	Enhanced AM activity of nisin and GSE	Sivarooban *et al.* (2008)
Soy protein isolate	Grape seed extract + EDTA	1% (w/w)	IN	Liquid or solid media	*E. coli O157:H7, S. typhimurium*, and *L. monocytogenes*	Reduced population of *E. coli O157:H7, S. typhimurium, L. monocytogenes*	Sivarooban *et al.* (2008)
Soy protein isolate	Nisin + EDTA	1×10^3 IU/g	IN	Liquid or solid media	*E. coli O157:H7, S.*	Reduced population of *E. coli O157:H7, S. typhimurium, L. monocytogenes*	Sivarooban *et al.* (2008)

Packaging Material	Antimicrobial Agent	Loading	Application[a]	Substrate	Microorganism(s)	Observations	References
					typhimurium, and *L. monocytogenes*		
Soy protein isolate films	Nisin	3-12 × 10^4 IU/15mL	IN	liquid culture media	*L. monocytogenes*	Inhibition against *L. monocytogenes* was concentration dependent	Ko *et al.* (2001)
Whey protein	Lactoperoxidase	0.01-0.4 (w/v)	IN	Agar and liquid culture media, smoked salmon	*L. monocytogenes*	Reduced population of *L. monocytogenes* by 3 log CFU g^{-1} on smoked salmon	Min *et al.* (2005a)
Whey protein	Malic acid	3% (w/v)	IN	Agar media	*L. monocytogenes*, *P. aeruginosa*, *P. commune*, *P. roqueforti* and *Y. lipolytica*	Inhibited *L. monocytogenes* and *P. aeruginosa*	Pintado *et al.* (2010)
Whey protein	Natamycin	2-5×10^{-3} g/mL	IN	Agar media	*L. monocytogenes*, *P. aeruginosa*, *P. commune*, *P. roqueforti* and *Y. lipolytica*	Inhibited *Y. lipolytica*, *Penicillium spp.*	Pintado *et al.* (2010)

Packaging Material	Antimicrobial Agent	Loading	Application[a]	Substrate	Microorganism(s)	Observations	References
Whey protein	Nisin	50 IU/mL	IN	Agar media	*L. monocytogenes*, *P. aeruginosa*, *P. commune*, *P. roqueforti* and *Y. lipolytica*	Inhibited *L. monocytogenes*	Pintado *et al.* (2010)
Whey protein isolate	Chitosan-lysozyme	3% (w/w)	C	Hard-boiled egg	*L. monocytogenes* and *S. enteritidis*	Ineffective against *L. monocytogenes* but reduced growth of *S. enteritidis*	Kim *et al.* (2008)
Whey protein isolate	Garlic oil	1-4% (w/v)	IN	Agar method	*E. coli O157:H7*, *S. aureus*, *S. enteriditis*, *L. monocytogenes*, and *L. plantarum*	Garlic oil inhibit *E. coli O157:H7*, *S. aureus*, *S. enteriditis*, *L. monocytogenes*, and *L. plantarum* at 3-4%	Seydim and Sarikus (2006)
Whey protein isolate	Grape seed extract	1.2-3.6 × 10^3 ppm	IN	Turkey frankfurter	*L. monocytogenes*, *E. coli O157:H7*, and *S. typhimurium*	Ineffective against *L. monocytogenes*, *E. coli O157:H7* but inhibited growth of *S. typhimurium*	Gadang *et al.* (2008)
Whey protein isolate	Malic acid	1.2-3.6 × 10^3 ppm	IN	Turkey frankfurter	*L. monocytogenes*, *E. coli O157:H7*, and *S.*	Ineffective against *L. monocytogenes*, *E. coli O157:H7* but inhibited growth of *S. typhimurium*	Gadang *et al.* (2008)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
					typhimurium		
Whey protein isolate	Nisin	6-18 × 10^3 IU/g	IN	Turkey frankfurter	*L. monocytogenes*, *E. coli O157:H7*, and *S. typhimurium*	Ineffective against *L. monocytogenes*, *E. coli O157:H7* but inhibited growth of *S. typhimurium*	Gadang *et al.* (2008)
Whey protein isolate	Oregano	1-4% (w/v)	IN	Agar method	*E. coli O157:H7*, *S. aureus*, *S. enteriditis*, *L. monocytogenes*, and *L. Plantarum*	Oregano demonstrated Inhibitory effect against *E. coli O157:H7*, *S. aureus*, *S. enteriditis*, *L. monocytogenes*, and *L. plantarum* at 3-4%	Seydim and Sarikus (2006)
Whey protein isolate	p-aminobenzoic acid	0.5-1.5% (w/v)	IN	Agar media	*L. monocytogenes*, *E. coli O157:H7*, and *S. Typhimurium DT104*	Inhibited *L. monocytogenes*, *E. coli O157:H7*, *S. Typhimurium*	Cagri *et al.* (2001)
Whey protein isolate	p-aminobenzoic acid	0.5-1% (w/v)	IN	Bologna summer Sausage	*L. monocytogenes*, *E. coli O157: H7*, and *S. Typhimurium DT104.*	Reduced *L. monocytogenes* by log 1.5-3.4 on bologna slices and increase by log 2.2 under control after 21 days. Population of *E. coli O157: H7* decrease by log 2.7-3.6	Cagri *et al.* (2002)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
Whey protein isolate	Rosemary	1-4% (w/v)	IN	Agar method	*E. coli O157:H7, S. aureus, S. enteriditis, L. monocytogenes,* and *L. plantarum*	Ineffective against all the reference microorganisms At all concentrations	Seydim and Sarikus (2006)
Whey protein isolate	Sorbic acid	0.5-1.5% (w/w)	IN	Agar media	*L. monocytogenes, E. coli O157:H7,* and *S. Typhimurium DT104*	Inhibited *L. monocytogenes, E. coli O157:H7, S. Typhimurium DT104*	Cagri *et al.* (2001)
Whey protein isolate	Sorbic acid	0.5-1% (w/v)	IN	Bologna and summer sausage	*L. monocytogenes, E. coli O157: H7,* and *S. Typhimurium DT104.*	Decreased population of *L. monocytogenes, E. coli O157: H7, S. Typhimurium*	Cagri *et al.* (2002)
Others							
Apple puree	Cinnamon	0.05-0.5% (w/w)	IN	Liquid culture	*E. coli O157:H7*	Film effective against E. *coli O157:H7*	Rojas-Grau *et al.* (2006)
Apple puree	Lemongrass oil	0.05-0.5%	IN	Liquid culture	*E. coli O157:H7*	Inhibited the growth of *E. coli O157:H7*	Rojas-Grau *et al.* (2006)

Packaging Material	Antimicrobial Agent	Loading	Applic-ation[a]	Substrate	Microorganism(s)	Observations	References
		(w/w)					
Apple puree	Oregano oils	0.05-0.1% (w/w)	IN	Agar media/solid media	*E. coli O157:H7*	Highly effective against *E. coli O157:H7*	Rojas-Grau *et al.* (2006)
PVOH, CTA, nylon 6,6	Lysozyme	10-300 mg/g	C	Liquid culture	*Micrococcus lysodeikticus*	All films demonstrated AM activity with nylon 6,6 showing the least effective	Appendini and Hotchkiss (1997)

2.4.1 The AM Activity of Polysaccharide Films Incorporated with AM agents

Biobased polysaccharides can be used for the production of biobased films. Polysaccharide-based films demonstrate adequate film-forming properties, although they are sensitive to moisture due to the hydrophilic groups in their structure (Baldwin *et al.*, 1995; Han & Floros, 1997; Krochta *et al.*, 1994). Phan *et al.* (2005) studied the functional properties of agar-based and starch-based films and their potential application in food packaging. They reported that films made from agar and cassava starch demonstrated advanced functional properties. However, these films exhibited poor moisture barrier properties compared to low-density polyethylene films because of the inherent hydrophilicity of the polysaccharides. Dias *et al.* (2010) developed biobased films based on rice starches that had improved mechanical properties.

Amongst the polysaccharide-based polymers, the starch-based ones are the most abundant and relatively inexpensive renewable materials. Starch is a natural polysaccharide primarily sourced from cereal grains, potatoes, tapioca and arrowroot (Baldwin *et al.*, 1995; Cutter, 2006; Zhang & Liu, 2009). Starch consists of amylose and amylopectin molecules present at different molecular ratios. Amylose is a linear molecule consisting of glucose units connected by 1,4-glucosidic linkages and amylopectin is a highly branched molecule consisting of short 1,4-glucose chains connected by 1,6-glucosidic linkages (Maizura *et al.*, 2007; Parker & Ring, 2001; Rodriguez *et al.*, 2003; Wu *et al.*, 1998). Starch is a semicrystalline, very hydrophilic material (Bicerano, 2003). The amorphous and crystalline phases affect the physical and chemical properties of starch-based films such as the mechanical and gas barrier properties (Cha & Chinnan, 2004; Liu, 2005). Films manufactured from starch-based

materials have better gas barrier properties (when dry) than synthetic polymer films but their mechanical properties are poorer. A high amylose starch polymer can be formed into consistent, relatively strong and flexible films that are highly impermeable to oxygen and carbon dioxide. This is in contrast to high amylopectin starch polymers, which can be formed only into non-continuous and brittle films (Cha & Chinnan, 2004; Gennadios *et al.*, 1997). As expected, starch alone cannot be formed into films with adequate properties for food packaging (Arvanitoyannis & Biliaderis, 1998a; Phan *et al.*, 2005). The intrinsic high level of hydrophilicity, poor mechanical properties and difficulties in processing limit its applications in food packaging unless modified mechanically, physically, chemically or genetically (Arvanitoyannis & Biliaderis, 1999; 1998b; Davis & Song, 2006; Garcia *et al.*, 2000a; Marron *et al.*, 2000; Tharanathan, 2003; Zhang & Liu, 2009). Several studies have demonstrated that modified starch-based materials can be used in commercial applications to package dry and other solid food products such as biscuits, snacks, cereals, fresh produce, fruits and vegetables (Avérous *et al.*, 2001; Bravin *et al.*, 2006; Cutter, 2006; Debeaufort *et al.*, 1998; Gennadios *et al.*, 1997; Nisperos-Carriedo, 1994; Wong *et al.*, 1994) and/or products with a low water activity (Olivas & Barbosa-Canovas, 2009). However, it is important to note that a particular issue of starch-based materials that needs to be considered when preparing films from these materials is their heat sealability. To address the issue of thermal resistance, starch-based materials can be modified chemically or physically (Liu *et al.*, 2009). After modifying the starch-based material such as converting it into a thermoplastic using a gelatinisation process, several conventional processing techniques such as extrusion, injection compression moulding and casting can be used to thermally

process starch-based material and thereby modifying it to produce a resultant heat sealable product.

Table 2.1 shows that many researchers have made considerable progress by successfully impregnating starch-based films with natural or synthetic AM agents. Such AM starch-based films have shown inhibitory activity to the growth of various microorganisms such as *S. enteritidis*, *L. plantarum*, *B. thermosphaceta B2*, and *L. monocytogenes*, *E. coli O157:H7*, *E. coli*, *S. aureus*, *S. typhimurium*. Durango *et al.* (2006) developed an AM film based on yam starch incorporated with chitosan at different concentrations (1%, 3% and 5% (w/v)) and reported a significant reduction of *S. enteritidis* in liquid culture by each of the films. Nam *et al.* (2007) incorporated 1% (w/w) lysozyme into a pea starch film and demonstrated an AM activity against *B. thermosphaceta B2*. Salleh *et al.* (2007) studied the synergistic effects of wheat starch films incorporated with lauric acid and chitosan and found a significant AM activity of these films against *B. subtilis* but not against *E. coli*. The authors claimed that starch-based films inhibited the growth of all tested microorganisms in liquid culture. The latter observation may be unrealistic in terms of the release of AM agent in the film because the starch-based film presumably dissolves in the liquid culture medium.

Baron and Sumner (1993) showed that starch films impregnated with potassium sorbate and acidified with lactic acid reduced the growth of *S. typhimurium* by 4 log CFU mL^{-1} after 2 h at 37°C. The population count of *E. coli O157: H7* decreased by 2 log CFU mL^{-1} after 3.5 h at 37°C. Furthermore, they found that corn-starch films impregnated with potassium sorbate inhibited the growth of *S. typhimurium* and *E. coli O157 H7* on

poultry products stored at 7°C for 12 days. Maizura *et al.* (2008) investigated the antibacterial activity of starch-alginate film incorporated with lemongrass oil. The AM film inhibited the growth of *E. coli O157:H7* and *S. enteritidis* determined by the agar disc diffusion assay but did not show any inhibitory effect on the growth of *S. aureus*. A recent study by Shen *et al.* (2010) found that a sweet potato starch film incorporated with 15% (w/w) potassium sorbate or 5% (w/w) chitosan showed a significant reduction of *E. coli* on solid and semi-solid media compared to a control film containing no potassium sorbate or chitosan that did not inhibit the growth of *E. coli*. The sweet potato starch film incorporated with 10% (w/w) chitosan suppressed the growth of *S. aureus*. Corrales *et al.* (2009) found that pea starch films impregnated with grape seed extract inhibited the growth of *B. thermosphaceta B2* on pork loin by 1.3 log CFU mL^{-1} within the first 4 days of storage at 4°C compared to the control film. Pelissari *et al.* (2009) investigated the AM activity of starch-based film incorporated with oregano essential oil (EO). The use of the AM starch-based film effectively inhibited the growth of *E. coli O157:H7*, *B. cereus* and *S. enteritidis* in the agar disc diffusion assay.

Many of the abovementioned studies demonstrated AM activity against various microorganisms using techniques involving agar-based and liquid culture media. Unfortunately, the question of the moisture sensitivity of the starch-based materials and the subsequent usefulness of their films as commercial packaging systems has not been adequately addressed in the technical/scientific literature. Therefore, further research is needed to show how to diminish the moisture sensitivity and to enhance the physico-mechanical properties of such starch-based materials so that these can be used also for packaging of moist food products. Although, many starch-based materials incorporated

with various AM agents demonstrate AM activity, an important aspect to be considered is the effect of increasing the concentration of the AM agent on the physico-mechanical properties of the resultant films. Shen *et al.* (2010) reported a deterioration in the physico-mechanical properties of films upon an increase in the potassium sorbate concentration**.** Indeed, such adverse effects could limit the prospects of applying these films in food packaging applications.

In many studies the AM activity of other polysaccharide-based materials such as chitosan incorporated with AM agents has been investigated. Chitosan films have exhibited inhibitory activity on the growth of various microorganisms, when impregnated with AM agents. For example, Ojagh *et al.* (2010b) developed chitosan films containing 0.4% to 2% (v/v) of cinnamon EOs and evaluated the AM efficacy of these films against *L. monocytogenes*, *L. plantarum*, *E. coli*, *L. sakei* and *P. fluorescens* in the disc diffusion assay. They reported that chitosan films containing these concentrations of cinnamon EOs inhibited the growth of all tested bacteria on agar media. Li *et al.* (2006) found that chitosan films incorporated with 463 international units (IU) of nisin inhibited the growth of *S. aureus*, *L. monocytogenes* and *B. cereus* using the agar diffusion method. However, nisin incorporated into chitosan film had no inhibitory effect against *E. coli*. The later observation is in agreement with the results of Pranoto *et al.* (2005b) who studied the AM effect of chitosan films impregnated with nisin at different concentrations against *E. coli*. The impregnated chitosan films were also tested against food pathogens including *S. aureus*, *S. typhimurium*, *L. monocytogenes* and *B. cereus*. In their findings, the AM chitosan film demonstrated inhibitory effects on *L. monocytogenes*, *S. aureus* and *B. cereus*. Increasing the

concentration of nisin in the film formulation did not improve the AM activity of the film. Ouattara *et al.* (2000b) found that chitosan films containing several organic acids (acetic and propionic) and cinnamaldehyde reduced the growth of *Enterobacteriaceae*, *Serratia liquefaciens* and *Lactobacillus sakei* on the surfaces of vacuum-packed cured meat products (bologna, cooked ham and pastrami) after a storage period of 21 days at 4°C. Duan *et al.* (2008) reported that chitosan films containing lysozyme demonstrated inhibitory activity against *E. coli* and *L. monocytogenes*. A significant release of lysozyme from the films was found. The storage conditions (time and temperature) did not affect the water vapour permeability of the film. Möller *et al.* (2004) studied the AM effectiveness of chitosan-hydroxypropylmethyl cellulose (HPMC) films, chitosan-HPMC films containing stearic and citric acids, and chemically modified chitosan-HPMC films. The chitosan-HPMC films, with and without stearic acid, reduced significantly the growth of *L. monocytogenes*.

Table 2.1 shows that other studies have evaluated the AM activity of AM agents incorporated into cellulose-based materials such as methylcellulose (MC) films. The cellulose-based materials are some of the naturally occurring polysaccharides with improved film-forming properties. Similarly to the starch-based materials, cellulose-based materials are hydrophilic in nature and have a semicrystalline structure and so are not generally suitable for packaging of moist food products (Baldwin *et al.*, 1995; Cutter, 2002). Many of the cellulose-based materials and/or their derivatives such as MC, HMPC and cellulose acetate are already manufactured commercially. The latter is widely used in packaging of baked goods and fresh food products (Weber, 2000). Although, there have been a limited number of studies conducted in the past using MC-

based materials and/or their derivatives, more recently there has been an increased recognition of the potential use of these materials in AM packaging systems for the preservation of food products against microbial contaminations and for the extension of the shelf life of the packaged products.

Several researchers have investigated the potential use of cellulose-based materials in AM packaging systems particularly in coating systems as discussed in Section 2.5. For example, Ayana and Nazan (2009) studied the antibacterial effectiveness of olive leaf extract incorporated into MC films against *S. aureus* in an agar disc diffusion test and on surfaces of Kasar cheese. The MC films demonstrated inhibitory activity against *S. aureus* on the agar medium. The films containing 1.5% (w/v) olive leaf extract decreased the population count of *S. aureus* on the surface of Kasar cheese by 1.22 log cycles after 14 days of storage. Santiago-Silva *et al.* (2009) investigated the AM activity of a cellulose-based film incorporated with pediocin. Using the challenge test on sliced ham inoculated with *L. innocua* and *Salmonella spp.* the AM cellulose-based film reduced the growth of *L. innocua* by 2 log cycles after 15 days of storage at 12°C. Similarly, the AM cellulose-based film effectively inhibited the growth of *Salmonella spp* by 0.5 log cycles after 12 days of storage.

Table 2.1 shows the AM activity of AM agents incorporated into other polysaccharide-based materials such as alginate, poly(lactic acid) (PLA) and pullulan-based films as determined by different researchers. Marcos *et al.* (2007) studied the effect of enterocins incorporated into a series of biobased films (alginate, zein and poly(vinyl alcohol)) for the preservation of ready-to-eat food products including sliced ham inoculated with *L.*

monocytogenes. These biobased AM films successfully delayed and/or reduced the growth of *L. monocytogenes* during storage at 6°C for 29 days. Recently, Jin and Zhang (2008) investigated a PLA film incorporated with nisin. They found that PLA containing nisin significantly inhibited the growth of *L. monocytogenes* in liquid culture and on liquid egg white. The PLA-nisin film was more active against the growth of *E. coli O157:H7* in orange juice than in liquid culture. Rojas-Grau *et al.* (2006) studied the antibacterial effectiveness of apple puree-based films impregnated with EOs (oregano, cinnamon and lemongrass) against *E. coli O157:H7*. All the evaluated films containing EOs were reported to be effective against *E. coli O157:H7* with the antibacterial activity of oregano oil notably higher than that of lemongrass and cinnamon oils. Kandemir *et al.* (2005) investigated the AM activity of pullulan-based films incorporated with partially purified lysozyme against the growth of *E. coli* and *L. plantarum*. The AM pullulan-based films were found to be effective against *E. coli* but did not show any AM activity against *L. plantarum*. Natrajan and Sheldon (2000b) evaluated the antibacterial effectiveness of calcium alginate and agar-based films incorporated with nisin against *S. Typhimurium* on broiler skin. Their results showed that the films containing nisin reduced the population of *S. Typhimurium*.

2.4.2 The AM Activity of Protein Films Incorporated with AM Agents

Proteins are biopolymeric materials that can be used for the production of biobased AM films as they have good film-forming properties. Protein-based polymers have amino acids in their monomer units. Packaging films have been manufactured from different proteins, such as corn zein, wheat gluten, soy protein, whey protein or their derivatives (Hernandez-Izquierdo *et al.*, 2008a). Packaging films made from protein-based

polymers possess adequate physico-mechanical properties (Krochta, 2002). Whey protein and corn zein incorporated with natural or synthetic AM agents have been extensively tested *in vitro* and on different food products against the growth of various microorganisms. A summary of the studies investigating the antibacterial effect of AM protein-based films is also presented in Table 2.1. Although these studies are not directly comparable in terms of the AM agents or microorganisms tested, the results demonstrate in general that whey protein isolate (WPI) films can be impregnated with AM agents and have the potential to be used as AM food packaging materials. However, no information could be found in the current technical/scientific literature on the cost/effective benefits of WPI-based films and therefore such information is needed before AM films from WPI-based materials for commercial applications could be efficiently manufactured.

Pintado *et al.* (2010) investigated the inhibitory effects of whey protein films incorporated with nisin, natamycin and malic acid against *P. aeruginosa, L. monocytogenes, Y. lipolytica, P. roqueforti, P. commune* using the agar disc diffusion method. They reported that whey protein films incorporated with AM agents demonstrated inhibitory effects against all tested microorganisms. Seydim and Sarikus (2006) tested the AM efficacy of WPI films incorporated with oregano, rosemary and garlic EOs against *E. coli O157:H7, S. aureus, S. enteriditis, L. monocytogenes,* and *L. plantarum.* The AM whey protein films containing oregano EOs at 2% (w/w) level demonstrated a higher inhibitory effect against the tested microorganisms than similar films containing garlic and rosemary extracts. Min *et al.* (2005a) investigated the AM effectiveness of whey protein films containing Lactoperoxidase against *L.*

monocytogenes using liquid and agar media as well as on smoked salmon. These films reduced the population of *L. monocytogenes* on smoked salmon by 3 log CFU g^{-1} after 35 days of storage compared to the control film. Gadang *et al.* (2008) evaluated the AM effectiveness of WPI films incorporated with a combination of nisin, malic acid, grape seed extract and EDTA against the growth of *L. monocytogenes*, *E. coli O157:H7*, and *S. typhimurium* inoculated on the surface of a turkey frankfurters. It was found that all the WPI films incorporated with the combination of AM agents decreased the population of *L. monocytogenes*, *E. coli O157:H7* and *S. typhimurium* on the surface of the turkey frankfurters by 3.2, 4.2 and 4.6 log CFU g^{-1} after 28 days of storage at 4°C compared to the control film.

Cagri *et al.* (2001) developed WPI films containing 0.5% to 1.5% (w/w) of sorbic acid (SA) or *p*-aminobenzoic acid (PABA) and evaluated the AM efficacy of these AM WPI films against *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium DT104* in a disc diffusion assay. They reported that WPI films containing 1.5% (w/w) PABA or SA inhibited the growth of *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium DT104* in that assay. These results were verified by Cagri *et al.* (2002) who examined the AM effectiveness of WPI films incorporated with 0.5% to 1.0% (w/w) PABA or SA against *L. monocytogenes*, *E. coli O157: H7* and *S. enterica subsp. Enterica serovar typhimurium DT104* inoculated on sliced bologna and summer sausage. Whey protein isolate films containing 1.5% w/w PABA or SA reduced the *L. monocytogenes*, *E. coli* and *S. enterica* population on both products after 21 days at 4 °C. Ko *et al.* (2001) studied the AM activity of WPI, SPI, egg albumin and wheat gluten films incorporated

with nisin against *L. monocytogenes*. They found that all these AM protein-based films inhibited *L. monocytogenes*.

Corn zein materials obtained from plant sources are an additional form of proteins that demonstrate good film-forming properties with the potential of being impregnated with AM agents in order to preserve food products from microbial contamination. Previous studies showed that corn zein films containing AM agents demonstrated AM activity against the growth of various microorganisms both *in vitro* and in various food products. In a detailed study by Hoffman *et al.* (2001) it was found that corn zein films incorporated with lauric acid, nisin, EDTA and combinations of these three compounds reduced *L. monocytogenes* in liquid culture, although there was no observed inhibitory effect in films incorporated with EDTA alone. All the films were reported to be bacteriostatic when a 10^4 CFU mL^{-1} *S. enteritidis* initial inoculum was used. Padgett *et al.* (1998) investigated the inhibitory effect of heat-pressed and cast corn zein films containing lysozyme and nisin and reported significant zones of inhibition for *Lactobacillus plantarum* by the cast film compared to the heat-pressed films. In another study Padgett *et al.* (2000) found an inhibitory activity of corn zein films incorporated with various levels of lauric acid and nisin on the growth of *L. plantarum* in liquid culture. Gücbilmez *et al.* (2007) developed AM films from corn zein incorporated with lysozyme and albumin proteins. They reported that these films demonstrated AM activity against the growth of *E. coli* and *B. subtilis*.

The AM activity of other types of protein-based films have been studied and reported in the scientific literature by different researchers (see Table 2.1). Kristo *et al.* (2008)

investigated the effectiveness of sodium caseinate (SC) incorporated with nisin, potassium or sodium lactate against *L. monocytogenes*. They found that SC films containing nisin exhibit the highest inhibitory effects on the growth of *L. monocytogenes* followed by films impregnated with potassium sorbate, whereas films containing sodium lactate were only slightly effective. Sivarooban *et al.* (2008) evaluated the AM properties of soy protein isolate (SPI) films containing 1% (w/w) of grape seed extract and nisin (1×10^3 IU g^{-1}). The AM SPI films demonstrated the greatest inhibitory activity against *L. monocytogenes* compared with the other tested systems. Oussalah *et al.* (2004) developed a protein-based edible film containing 1% (w/w) oregano and pimento EOs or a mixture of both EOs and evaluated the AM effects of these films on the preservation of whole beef muscle. The results showed an effectiveness of the AM films against *Pseudomonas spp.* and *E. coli 0157:H7* inoculated on the surface of the beef. Their results also suggested that films containing oregano EO were more effective against the growth of both microorganisms compared to films containing pimento.

2.5 The AM Activity of Biobased Films Coated with AM Agents

In addition to the direct incorporation of AM agents into packaging films discussed above, AM agents can be coated on the surface of packaging materials in order to provide a high concentration of the agent in contact with the surface of food product (An *et al.*, 2000; Gennadios *et al.*, 1997). The application of an AM agent on a packaging material can be achieved by using various coating techniques including immersion of the substrate or by spraying the substrate with a coating/carrier solution.

For this purpose, the AM agent is dissolved in an appropriate solvent such as water, ethanol or isopropanol before applying it to the packaging material (Krochta, 2002). Little has been reported on the activity of AM agents coated on biobased polymers. Some of the relevant studies are given in Table 2.1.

Miltz *et al.* (2006) studied the effectiveness of a corn starch-based film coated with the peptide dermaseptin S4 derivative as an AM agent against moulds and aerobic bacteria on cucumbers. They reported that this system was very effective. Coma *et al.* (2001) found that cellulose films coated with nisin inhibited *L. innocua* and *S. aureus* on laboratory media. Chen *et al.* (1996) prepared AM films containing 2% or 4% (w/w) of sodium benzoate and potassium sorbate by casting MC, chitosan and their mixtures. They evaluated the antimycotic activity of the AM films against *Rhodotorula rubra* and *Penicillium notatum* and found that MC and MC/chitosan films containing 2% and 4% (w/w) sodium benzoate and potassium sorbate respectively inhibited the growth of these microorganisms. Ming *et al.* (1997) reported that a cellulose casing coated with pediocin inhibited completely the growth of *L. monocytogenes* on ham, turkey breast and beef products compared to the control film after 12 weeks of storage at 4°C. Janes *et al.* (2002) investigated the AM effect of corn zein films coated with nisin and/or 1% (w/w) calcium propionate against *L. monocytogenes* inoculated on ready-to-eat chicken samples and found that the coated films inhibited the growth of the microorganism. Kim *et al.* (2008) evaluated recently the AM effectiveness of chitosan and WPI coated with lysozyme against the growth of *L. monocytogenes* and *S. enteritidis* inoculated on hard-boiled eggs. The Chitosan-lysozyme system controlled the growth of *S. enteritidis* on hard-boiled shell-on and on peeled eggs. Siragusa and Dickinson (1993; 1992) found

that calcium alginate coatings and films containing organic acids effectively reduced the population of *L. monocytogenes*, *S. typhimurium* and *E. coli O157:H7* on the surface of beef carcass.

2.6 The AM Activity of Biobased Films Immobilised with AM Agents

Effective AM packaging systems can also be achieved by the immobilisation of an AM agent in a polymeric material. According to Steven and Hotchkiss (2003), the AM agents that can be immobilised include peptides, proteins or enzymes. These agents can be synthesised on the surface or extracted separately and then covalently linked to the polymer substrate. An AM agent that is covalently immobilised onto the packaging material is not released but becomes effective in inhibiting microbial growth when in contact with the surface of the packaged food product (Han, 2003). Different studies have been conducted focusing on immobilisation of AM agents onto packaging materials. Appendini and Hotchkiss (1997) investigated the efficiency of lysozyme immobilised on polyvinyl alcohol (PVOH) beads, nylon 6,6 pellets and cellulose triacetate (CTA) films. They reported that the viability of *Micrococcus lysodeikticus* was reduced in the presence of immobilised lysozyme on CTA film that was found to show the highest AM activity amongst the studied structures. Cutter and Siragusa (1997) assessed the potential decontamination of raw beef by applying organic acids (lactic or acetic acid) immobilized onto calcium alginate films. They reported a considerable reduction of *L. monocytogenes* growth with the treated films compared to a calcium alginate film without acid treatment. Cutter and Siragusa (1996) studied the AM activity of nisin immobilised onto calcium alginate films against *Brochothrix*

thermosphacta on beef surfaces. They found that calcium alginate films treated with nisin suppressed the growth of *B. thermosphacta* by 2.42 log CFU cm^{-2} after 7 days compared to an untreated film. A greater and steady nisin activity was found when the tissues were ground and stored under refrigerated conditions in the AM immobilized film for up to 7 days compared to the use of sprayed nisin.

2.7 Effects of Additives on the Properties of Biobased Films

Along with the increase in the potential uses of biobased films, packaging materials with suitable physico-mechanical properties can be prepared from renewable biopolymeric materials mainly from polysaccharides and protein-based materials (Baldwin *et al.*, 1995; Krochta *et al.*, 1994; 1997). Polysaccharides and protein-based materials in particular exhibit adequate film properties such as low gas permeability (Hernandez-Izquierdo & Krochta, 2008b; Robertson, 2007a). Adequate physico-mechanical properties for such biodegradable films or coated films are essential to ensure that the film has sufficient strength and integrity during transportation, handling and storage of the packaged foods (Ozdemir & Floros, 2008a). To date, manufacturing of biopolymeric films with adequate moisture barrier properties comparable to those of synthetic packaging materials remains a challenge because of the hydrophilic nature of many biopolymers (Weber *et al.*, 2002). There is growing evidence that the physical, mechanical and barrier properties of AM films may be affected by additives such as AM agents that are added to the packaging material (Han, 2003; 2005a), the type and amount of plasticiser used during film processing (Da Róz *et al.*, 2006; Talja *et al.*, 2007), the moisture content and water activity of the contained food product (Chaléat *et al.*, 2008; Rico-Peña & Torres, 1991), the relative humidity (RH) in the environment

(Myllärinen *et al.*, 2002a; Russo *et al.*, 2007; Stading *et al.*, 2001) and that the addition of lipids increases moisture barrier properties of films (Chick & Hernandez, 2002; Garcia *et al.*, 2000a; Han *et al.*, 2006; Sebti *et al.*, 2002; Shellhammer & Krochta, 1997). This subsection of the review provides a detailed overview of each of the factors that may affect the performance characteristics of biopolymers used as food packaging materials with an emphasis on AM agents and plasticisers. This is followed by a consideration of the effect of lipids on the water vapour permeability (WVP) and gas permeability of packaging films.

2.7.1 Effects of Antimicrobial Agents on the Properties of Biobased Films

In AM packaging systems, both synthetic and natural AM agents can be incorporated into or coated onto the packaging material. Packaging materials can be impregnated with various AM agents by blending the AM agent with a base polymer before extrusion or compression moulding into films (Mistry, 2006; Rardniyom *et al.*, 2008b; Rupika, 2008a; Suppakul *et al.*, 2006). According to Suppakul (2004), an AM agent can be dispersed evenly in the amorphous region of the polymeric material. Even though the impregnation of AM agents into the base polymer is one of the feasible means of achieving good AM activity of the film, the physico-mechanical properties of the films are important aspects to be considered when designing the AM film for food packaging applications (Ozdemir & Floros, 2008a). Many AM agents have exhibited AM activity against various foodborne pathogens such as *Listeria*, *S. aureus*, *E. coli* and *Salmonella.* However, their direct incorporation into/onto the film during extrusion/compression and/or coating at high concentrations may affect their physico-mechanical and/or barrier

properties (Cooksey, 2000; Han, 2005a). Table 2.2 summarises the effects of AM agents on the properties of polysaccharide and protein-based AM films as reported by different researchers. Table 2.2 also lists the effect of plasticisers and/or lipids on the properties of such films.

Table 2.2 Effects of Additives on Properties of Biobased Films

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
Apple puree	Cinnamon	0-0.5% (w/w)	Incorporation of 0.05-0.1% (w/w) slightly increases TS	Slight increase for 0.05-0.1 but decrease for 0.5 from 5.06-4.49	Presence of cinnamon did not change the elongation at break but 0.5 % (w/w) increase elongation at break compared to control film	WVP decreases from 7.48 to 6.82 gmm kPa^{-1} h^{-1} m^{-2}	Rojas-Grau *et al.* (2006)
Apple puree	Lemongrass oil	0-0.5% (w/w)	Addition of 0.05-0.1% significantly decrease the TS	Lemongrass oil slightly decreased Young's modulus	Did not demonstrate discernible effect when compared with control film	Slightly decreased WVP from 6.71 to 6.62 gmm kPa^{-1} h^{-1} m^{-2}	Rojas-Grau *et al.* (2006)
Apple puree	Oregano oils	0-0.1% (w/w)	Did not demonstrate effect on TS (0.64-0.62)	Oregano oils slightly decreased from Young's modulus 5.06 to 4.73	elongation at break slightly increased from 25 to 26.5	Decrease WVP from 7.04 to 6.17 gmm kPa^{-1} h^{-1} m^{-2}	Rojas-Grau *et al.* (2006)
Cellulose,	Potassium	4% (w/v)	Has TS 3.8	-	Has elongation at	-	Chen *et al.* (1996)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
chitosan	sorbate		compared with 2.8 of control film		break of 28.5 compared with 19.6 for the control film		
Cellulose, chitosan	Sodium benzoate	4% (w/v)	Has TS 3.0 compared with 2.8 of control film	-	Has elongation at break of 22.5 compared with 19.6 for the control film	-	Chen *et al.* (1996)
Chitosan	Cinnamon oil	0.4-2% (v/v)	Increases TS from 13.35 to 29.23	-	elongation at break decreases from 16.57 to 3.58	Decreases WVP from 1.352 to 1.003	Ojagh *et al.* (2010a)
Chitosan	Oregano EOs	1-4% (w/w)	Addition of oregano EOs decrease TS (18-106)	Addition of oregano EOs decrease TS -	Addition of oregano EOs increase elongation at break of the film From 5 to 20	WVP decreases with increasing concentration of oregano EOs in the film matrices (14-80)	Zivanovic *et al.* (2005)
Chitosan-HMPC	Stearic acid		increases from 24 to 30	increases Young's modulus from 18-31	Decreases elongation at break from 3.87 to 1.79	-	Möller *et al.* (2004)
HMPC	Glycerol	10-50% (w/w)	decrease TS from 63 ± 8 to 16 ± 3 MPa	reduce Young's modulus from 2334 ± 99 to 421 ± 16 MPa	glycerol increase the elongation at break from 13 ± 1% to 50 ± 6%	WVP glycerol double from 4.2×10^{-10} to 8.8×10^{-10} g m^{-1} s^{-1} Pa^{-1}	Imran *et al.* (2010)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
HMPC	Nisin	10^4 IU	Decrease tensile strength from 63 to 43 MPa	Decrease Young's modulus from 2334 to 856 MPa	Increase the elongation at break from 13 to 26	WVP increased from 4.2×10^{-10} to 4.9×10^{-10} g m^{-1} s^{-1} Pa^{-1}	Imran *et al.* (2010)
Sodium caseinate	Potassium sorbate	10-25 (w/w)	Addition of Potassium sorbate induced significant reduction in the Tensile strength values	The Young's modulus values have been reduced significantly by the addition of Potassium sorbate	Film flexibility increases with the increases in concentration of Potassium sorbate in the film	increased WVP from 10×10^{10} to 22.8×10^{10} g s^{-1} m^{-1} Pa^{-1}	Kristo *et al.* (2008)
Sodium caseinate	Sodium lactate	10-40 (w/w)	The tensile strength values decrease from 68 to 38MPa when 10% is added	The addition of 10% Na la decrease tensile strength from 2443 to 1400 MPa	The addition of Na lactate caused an increase in the elongation at break	increased WVP from 10×10^{10} to 65.3×10^{10} g s^{-1} m^{-1} Pa^{-1}	Kristo *et al.* (2008)
Starch	Beeswax	0-40% (w/w)	Decrease tensile strength from 2.3 to 1.8	Increases from 12.1 to 41.0	elongation at break decreases from 51.4 to 23.95	Decreases WVP from 7.78 to 6.56 gmm kPa^{-1} h^{-1} m^{-2}	Han *et al.* (2006)
Starch	Cinnamon	0.15 % (w/w)	tensile strength lower than that of the control film		elongation at break was significantly lower than that of	Cinnamon reduces WVP of film	Kechichian *et al.* (2010)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
					the control film		
Starch	Clove	0.15 % (w/w)	tensile strength lower than that of the control film	-	elongation at break was observed to be lower than that of the control film	Clove incorporated into film reduces WVP	Kechichian *et al.* (2010)
Starch	Fructose	1.2-3 g/g	Decrease tensile strength from 8.4-1.9	decreases Young's modulus from 188.6-9.4	elongation at break increases from 30.3 to 79.3	increases WVP from 1.96 to 4.78 gmm kPa^{-1} h^{-1} m^{-2}	Zhang & Han (2006)
Starch	Glucose	1.2-3 g/g	Decrease tensile strength from 6.6-3.8	decreases Young's modulus from 110.9 to 7.8	elongation at break increases from 54.6 to 76.2	increases WVP from 1.9 to 3.88 gmm kPa^{-1} h^{-1} m^{-2}	Zhang & Han (2006)
starch	Glycerol	20-45% (w/w)	Increase of glycerol decrease tensile strength 21.7 to 5.4	Addition of glycerol resulted in the decrease of Young's modulus values (0.2-40.5)	The elongation at break of film increase from 5.2 to 153.2	When concentration of glycerol is used, WVP values tended to increased from 2.4 × 10^{-9} to 4.9 × 10^{-9} g m^{-1} s^{-1} Pa^{-1}	Alves *et al.* (2007)
Starch	Glycerol	1.2-3 g/g	Decrease tensile strength from 5.8-1.4	decreases Young's modulus from 97-5-7.8	elongation at break increases from 37.6 to 46.4	increases WVP from 2.75 to 9.97 gmm kPa^{-1} h^{-1} m^{-2}	Zhang & Han (2006)
Starch	glycerol	20% (w/w)	Addition of	-	Caused increase of	Glycerol increased	Rodriguez *et al.* (2006)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
			glycerol decreased tensile strength from 44.1 to 20.3		elongation at break from 5.9 to 12.1	WVP of film 1.5 to 2.2 g mm kPa^{-1} h^{-1} m^{-2}	
Starch	Mannose	1.2-3 g/g	Decrease tensile strength from 6.3-3.9	decreases Young's modulus from 111.2 to 23.6	mannose increases elongation at break from 70.2 to 145.2	increases from 2.27 to 4.63 gmm kPa^{-1} h^{-1} m^{-2}	Zhang & Han (2006)
Starch	Potassium sorbate	0.3% (w/w)	Tensile strength decreases to 0.74 compared with control with 2.35	Film with sorbate has low Young's modulus of 7.6 compared with 29.0 of control film	-	Film with sorbate has WVP of 6.1×10^{10} compared with 6.3×10^{10} g m^{-1} s^{-1} Pa^{-1} of control film	Flores *et al.* (2007a)
Starch	Sorbitol	1.2-3 g/g	Decrease from 5.7-1.2	decreases Young's modulus from 106.6 to 7.1	Sorbitol increases elongation at break from 61.1 to 68	increases WVP from 2.61 to 6.7 gmm kPa^{-1} h^{-1} m^{-2}	Zhang & Han (2006)
Starch	Sorbitol	15-30% (w/w)	Increase of sorbitol decrease tensile strength 4.8 to 1.1	Addition of sorbitol caused reduction of Young's modulus values (219-59)	The elongation at break of film increased from 10 to 28	-	Da Róz *et al.* (2006)
Starch-	glycerol	20% (w/w)	The addition of	-	Glycerol induced	Glycerol decreases	Maizura *et al.* (2007)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
alginate			glycerol reduced the tensile strength from 41.6 to 16.0		flexibility of film with elongation at break of 3.7 compared with 1.7 of control film	WVP from 5.1×10^{-10} to 2.4×10^{-10} g.m m^{-2} s^{-1} Pa^{-1}	
Starch-alginate	Lemongrass oil	0-0.4% (v/w)	Addition of lemongrass oil at increasing concentration level caused reduction of tensile strength from 41.6 to 32.3	-	lemongrass oil increases film elongation at break from 1.7 to 2.0	lemongrass oil increases WVP from 4.1×10^{-10} to 5.1×10^{-10} g.m m^{-2} s^{-1} Pa^{-1}	Maizura *et al.* (2007)
Starch-chitosan	Oregano EOs	0.1-1% (w/w)	Decrease tensile strength from 1.96 to 1.43	Decrease Young's modulus from 67.72 to 18.9	Increase elongation from 27.18 to 48.4	decreases WVP from 0.99×10^{-10} to 0.62×10^{-10} g m^{-1} s^{-1} Pa^{-1}	Pelissari *et al.* (2009)
Starch-MC	glycerol	5-30% (w/w)	tensile strength decreased from 59.4 to 33.5 when concentration of glycerol was increased.	-	Elongation at break increased from 11.9 to 34.2%	The WVP values increases from 3.2×10^{-11} to 20.7×10^{-11} g m^{-1} s^{-1} Pa^{-1} compared with the control film (0.48 ×	Arvanitoyannis & Biliaderis (1999)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
						10^{-11} g m^{-1} s^{-1} Pa^{-1})	
Starch-MC	Sorbitol	5-30% (w/w)	tensile strength decreased from 54.6 to 28.4 when concentration of sorbitol was increased.	-	Elongation at break increased from 14.3 to 39.4%	WVP increases from 4.5 × 10^{-11} to 26.5 × 10^{-11} g m^{-1} s^{-1} Pa^{-1} compared with control film (0.48 × 10^{-11} g m^{-1} s^{-1} Pa^{-1})	Arvanitoyannis & Biliaderis (1999)
Starch-MC	Water	5-30% (w/w)	tensile strength decreased from 73.1 to 38.7 when concentration of water was increased.	-	Elongation at break increased from 4.0 to 24.5%	-	Arvanitoyannis & Biliaderis (1999)
Starch-MC	Xylose	5-30% (w/w)	tensile strength decreased from 48.4 to 25.6 when concentration of xylose was increased.	-	Elongation at break increased from 8.8 to 28.4%	The WVP values increases from 4.9 × 10^{-11} to 28.7 × 10^{-11} g m^{-1} s^{-1} Pa^{-1}	Arvanitoyannis & Biliaderis (1999)
Tomato-based	Carvacrol	0-1.5% (w/w)	decreases tensile strength from 11.4 to 8.9	Slightly decreases Young's modulus from 248.1 to 187.2	Elongation at break slightly increases from 11.2 to 11.6	Increases from 2.44 to 2.61 gmm kPa^{-1} h^{-1} m^{-2}	Du *et al.* (2008a)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
				for 0, 1.5% respectively. However increases of Young's modulus from 310.3 to 259.9 was observed for 0.5 and 1.0% respectively	for 1.5%. However 0.5 and 1.0% have been reported to have decreased elongation at break.		
Whey protein	Potassium sorbate	10% (w/w)	Tensile strength decreased from 3.32 to 2.65	Addition of sorbate caused significant increase of Young's modulus from 83.97 to 88.35	elongation at break increase when film was incorporated with 0.5 and 1.0% (from 27.6 to 78.6)	-	Ozdemir & Floros (2008a)
Whey protein isolate	p-aminobenzoic acid	0.5-1.5% (w/v)	tensile strength slightly decreased from 5.88 to 5.80	-	elongation at break Increases from 6.37 to 74.8	Increases from 27.24 to 56.95 gmm kPa^{-1} d^{-1} m^{-2}	Cagri *et al.* (2001)
Whey protein isolate	Sorbic acid	0.5-1.5% (w/w)	Decreases tensile strength from 5.88 to 2.6	-	elongation at break increases from 6.37 to 42.16	Increases WVP from 27.24 to 45.55 gmm kPa^{-1} d^{-1} m^{-2}	Cagri *et al.* (2001)

Film	Additive	Loading	Tensile strength (MPa)	Young's modulus (MPa)	%Elongation at break	Water vapour permeability	References
WPI	Glycerol	30-50% (w/w)	tensile strength is reduced from 10 to 4	Young's modulus diminished from 251 to 60	Addition of glycerol caused changed of elongation at break from 43 to 94	-	Sothornvit *et al.* (2007)
WPI	Glycerol	33.3-41.18% (w/w)	Increasing level of glycerol decrease the tensile strength of the film (4.26 to 3.7	Increasing level of glycerol caused significant decrease of Young's modulus (159.49 to 68.67)	Addition of glycerol resulted in the increases of elongation at break for the film (33.63 to 50.78)	The WVP increased from 115.75 to 140.52 gmm kPa^{-1} d^{-1} m^{-2}	Shaw *et al.* (2002)
WPI	Oregano	0-1.5%(w/w)	decreases tensile strength	decreases Young's modulus	Increases elongation at break	Oregano at 1-1.5% w/w increases WVP from 8.6 to 11.0 g mm kPa^{-1} h^{-1} m^{-2}	Zinoviadou *et al.* (2009)
WPI	Soya oil	10.77-21.05% (w/w)	Increasing level of oil decrease tensile strength of film 4.26 to 4.06	Increasing level of oil decrease Young's modulus 59.49 to 105.29	Soya oil increases of elongation at break of film (33.63-44.05)	There was increase of WVP from 115.75 to 126.41 gmm kPa^{-1} d^{-1} m^{-2}	Shaw *et al.* (2002)

Various studies have investigated the effects of common food preservatives such as potassium sorbate and sodium benzoate on the properties of packaging films (see Table 2.2). For example, in a recent study by Ozdemir and Floros (2008a) the effect of potassium sorbate on the mechanical properties of whey protein films was investigated. The authors reported that 10% (w/w) potassium sorbate: (i) decreased the tensile strength of the film from 3.32 to 2.65 MPa, (ii) caused a significant decrease in the Young's modulus (23.7MPa) compared with 84.0 MPa of the control film and (iii) caused an increase in the elongation at break (78.6%) compared to the control film (27.6%). Ozdemir and Floros (2008b) investigated the effect of potassium sorbate in whey protein films on their water vapour barrier. The incorporation of 10% (w/w) potassium sorbate into whey protein film was found to increase slightly the water vapour permeability (WVP) of the film. Flores *et al*. (2007a) studied the effects of incorporating potassium sorbate on the properties of tapioca starch-based films. It was reported that the potassium sorbate: (i) affected the film's solubility and colour, (ii) decreased the tensile strength (from 2.35 to 0.74 MPa) and Young's modulus (from 29.0 to 7.6 MPa) of the film compared with the control film. The latter observation has been supported by a recent study by Shen *et al.* (2010) showing that in a sweet potato starch film incorporated with 15% (w/w) potassium sorbate there was a decrease of the tensile strength and elongation at break compared to the control film. Conversely, the incorporation of 15% (w/w) potassium sorbate increased the WVP, oxygen permeability and the solubility of the film in water.

Kristo *et al.* (2008) investigated the effect of sodium lactate and potassium sorbate incorporated into sodium caseinate (SC) films on the permeability and mechanical properties of the films. They reported that the water content and the WVP of SC films increased with increasing sodium lactate and potassium sorbate concentration, with sodium lactate-

containing SC films showing a higher capacity to absorb moisture and a greater WVP than the films containing potassium sorbate. In contrast, the addition of nisin did not cause significant changes in the water uptake and the WVP of the SC films. An increase in the sodium lactate and potassium sorbate concentration resulted in a reduction of Young's modulus and tensile strength of the films. Conversely, an increase in the sodium lactate and potassium sorbate concentration increased the elongation at break. The effect of AM agents on the properties of starch films was investigated by Ofman *et al*. (2004) who studied the effect of various concentrations of sodium benzoate or potassium sorbate impregnated into tapioca starch films on the water sorption and mechanical properties. The incorporation of sodium benzoate or potassium sorbate into starch matrices prior to gelatinisation was found to increase the amount of water sorbed by the starch-based film. Chen *et al*. (1996) studied the effects of sodium benzoate and potassium sorbate on the physicochemical properties of chitosan-methylcellulose films. They found that sodium benzoate and/or potassium sorbate at 4% (w/v) caused an increase in the tensile strength from 2.8 to 3.0 MPa and 3.8 MPa respectively compared to the control film. Addition of sodium benzoate or potassium sorbate at a concentration of 4% (w/v) increased the elongation at break of the chitosan-MC film from 19.6% to 22.5% and 28.5% respectively compare to the control film.

Many studies have examined the effects of natural AM agents on the properties of AM films as shown in Table 2.2. Zinoviadou *et al*. (2010) indicated that oregano essential oils (EOs) incorporated into WPI films caused a decrease in the tensile strength and Young's modulus. The authors also reported that EOs added to WPI films increases the elongation at break when the concentration was increased from 0.5 to 1.5% (w/w). Zivanovic *et al*. (2005) studied the effects of oregano EOs on the properties of chitosan films. Their results showed that chitosan films containing oregano EOs caused a decreased tensile strength but an

increased elongation at break of the films. These results were confirmed by the results of a recent study conducted by Pelissari *et al*. (2009) investigating the effects of varying concentration of oregano EOs on the properties of starch-chitosan films. They found that the incorporation of oregano EOs decreased the tensile strength (from 1.96 to 1.43 MPa), Young modulus (from 67.7 to 18.9 MPa) and the WVP (from 0.99 to 0.62) of the films compared to the control. In contrast to the above observations, the addition of oregano EOs at varying concentration increased the elongation at break of the starch-chitosan film (Pelissari *et al.*, 2009). Pruneda *et al*. (2008) incorporated Mexican oregano into soy protein isolate films plasticised with various polyols and reported an increase in the WVP of the films and a dark reddish appearance. Du *et al.* (2008a) examined the physico-mechanical properties of tomato-based films containing carvacrol. The incorporation of carvacrol at a level of 1-1.5% (w/w) showed a decrease in the tensile strength in range of 11.1 to 8.9 MPa compared with a control film (11.4 MPa). The researchers found that increasing the concentration of carvacrol from 0 to 1.0% (w/w) resulted in a reduction in the elongation at break (from 10.7 to 9.5%) in the film. In addition, the incorporation of 0.5 to 1.5% (w/w) carvacrol into the tomato-based film increased the WVP.

Ojagh *et al*. (2010a) developed chitosan films containing 0.4% to 2% (v/v) of cinnamon EOs and evaluated their effect on the physical and mechanical properties of the films. They reported that chitosan films containing 2% (v/v) of cinnamon EOs increased the tensile strength to 29.23 MPa compared to 10.97 MPa of the control film. In contrast, the elongation at break for the same film decreased from 24.7% to 3.5%. Kechichian *et al*. (2010) investigated the effect of clove and cinnamon on the properties of cassava starch film. The addition of 0.06% (w/w) clove and cinnamon EOs decreased the tensile strength from 4.7 to 2.2 MPa compared to the control film. Rojas-Grau *et al*. (2006) studied the effects of EOs

(oregano, cinnamon and lemongrass) on the physicochemical properties of apple puree-based films. They found that increasing the concentration of lemongrass EOs from 0.05 to 0.1% (w/w) reduced the tensile strength of the film compared to the control. However, the addition of an equal amount of cinnamon EOs increased the tensile strength of the film. Their results showed that there was no significant effect on the elongation at break of the films containing the EOs except for a film containing cinnamon EOs at a level of 0.5% (w/w). There was no clear effect of the EOs incorporated into the film on the elastic modulus except for the case of cinnamon EOs at a level of 0.075% (w/w) that increased the elastic modulus. When oregano, cinnamon and lemongrass EOs were incorporated into the film, there was a significant decrease in the WVP.

Durango *et al*. (2006) observed a lower transparency of yam starch films incorporated with chitosan but these films showed high tear resistance. The flexibility of yam starch films was similar to that of an LDPE film with a thickness of 25 µm. Corrales *et al*. (2009) reported a decrease in the puncture resistance and tensile strength of pea starch films impregnated with grape seed extract (GSFE) compared to the control film. Imran *et al*. (2010) found that nisin incorporated into HMPC film decreased the tensile strength and Young's modulus of the film compared to the control. On the other hand the incorporation of nisin and glycerol into HMPC caused a 50% increase in the elongation at break of the film. Maizura *et al.*(Maizura *et al.*, 2007) found that lemongrass oil impregnated into starch-alginate at the concentration range of 0-0.4% (v/w) reduced the tensile strength of the film from 41.6 to 32.3 MPa. However, there was a slight increase in the elongation at break (from 1.7 to 2.0%). Furthermore, it was found that the impregnation of starch-alginate films with lemongrass oil did not have a significant effect on the WVP of the films.

The overall trend shown in Table 2.2 demonstrates that several AM agents tend to lower the tensile strength and Young's modulus with increasing their concentration in the film. However, a vast number of studies reported an increase in the flexibility of many films with an increase in the concentration of AM agents. Nonetheless, the effect that AM agents have on the properties of biobased films is not directly comparable because many studies used different AM agents and different polymers. Unfortunately, none of the above mentioned studies recommended an optimum concentration of AM agents to be integrated into the packaging material. Table 2.2 shows that in many studies, the AM efficacy against various microorganisms was found at low concentrations. Thus, the inhibitory effects of the AM agents at low concentrations delineate the importance of a cost analysis to be made when considering utilisation in commercial AM packaging systems.

2.7.2 Effects of Plasticisers and RH on Properties of Biobased Films

Plasticisers are relatively low molecular weight compounds that can be copolymerised with the polymer or added to it in order to reduce the intermolecular and intramolecular forces and increase the mobility of the polymeric chains (Garcia *et al.*, 2000b; Sothornvit & Krochta, 2005; Tharanathan, 2003). Plasticisers are usually mixed with biopolymers such as starch-based materials to aid processing, improve film flexibility and lower the glass transition temperature of the polymer (Arvanitoyannis & Biliaderis, 1999; Avérous *et al.*, 2000; Brody, 2005; Fang *et al.*, 2005; Kim *et al.*, 2002a; Krochta, 2002; López *et al.*, 2008; Zhang & Liu, 2009). The type and amount of plasticiser, its number of functional hydroxyl groups and its compatibility with the polymer may affect the properties of the resultant films (Cuq *et al.*, 1997; Da Róz *et al.*, 2006; Talja *et al.*, 2007; Yang & Paulson, 2000). Examples of plasticisers that are commonly used with biopolymers include polyols such as glycerol,

sorbitol and mannitol, monosaccharides such as fructose, glucose and mannose, and polyethylene glycol (Brody, 2005; Kester & Fennema, 1986). Water is another important plasticiser for biobased films although moisture may affect the film properties (Krochta, 2002; Van Soest & Essers, 1997). Water can be added to a starch-based film in order to break its native granular structure and hydrogen bonding (Mali *et al.*, 2002; Myllärinen *et al.*, 2002b; Yang & Paulson, 2000). When a high concentration of plasticiser is used, the mechanical strength, barrier properties and rigidity are decreased (Gontard *et al.*, 1993). According to McHugh and Krochta (1994) and Sothornvit and Krochta (2000), plasticisers that are added to film formulations decrease the film density and increase the free volume of the film matrix which, in turn, increases the permeability of the films to gases and vapours. For example, glycerol molecules interfere with starch packing, thus decreasing intermolecular attraction and increasing polymer mobility (Garcia *et al.*, 2000a).

As shown in Table 2.2, many researchers have undertaken a detailed study of the plasticising effects of various polyols (sorbitol, glycerol); monosaccharides sugar (fructose, glucose, and mannose) and water in various packaging, particularly biodegradable, materials such as polysaccharide-based films. The plasticising effects of various polyols, monosaccharides and water in a pea starch film has been studied by Zhang and Han (2008). They found that the moisture content of the film was significantly affected by the RH of the environment but only slightly by the concentration of the monosaccharides. Films plasticised with glycerol had the highest moisture content, demonstrating that water molecules play a greater role in plasticising starch films than glycerol. Films plasticised with monosaccharides and polyol-plasticised films had a similar tensile strength. In contrast, monosaccharide-plasticised films had higher elongations and lower modulus of elasticity values than polyol-plasticised films, signifying that monosaccharides can improve the mechanical properties of starch films. An

increase in monosaccharide and/or polyol concentration resulted in an increase in WVP. In a similar study, Zhang and Han (2006) reported that films plasticised with a monosaccharide (mannose, glucose and fructose) had a higher tensile strength and elongation, and a lower WVP than the films plasticised with polyols. The monosaccharide-plasticised films had a similar modulus of elasticity to the polyol-plasticised films and the latter films had a lower glass transition temperature than the former ones.

Róz *et al*. (2006) investigated the effect of several plasticisers including sorbitol on the properties of processed thermoplastic starch film. They reported that doubling the concentration of sorbitol from 15 to 30% (w/w) resulted in a significant decrease in the tensile strength (from 4.8 to 1.1 MPa). The study also found a decrease in the Young's modulus from 219 to 59 MPa when the sorbitol concentration was increased from 15 to 30% (w/w). As expected, the elongation at break of plasticised starch films increased with increasing concentration of sorbitol. Talja *et al*. (2007) found that glycerol, xylitol and sorbitol affected the physico-mechanical properties of potato starch films. They reported that the WVP of the films increased with increasing plasticiser content and storage RH; increasing RH caused a reduction in both the Young's modulus and the tensile strength of the film. Increasing the plasticiser content resulted in an increase in the elongation at break for all plasticiser types. The authors observed that the effects of plasticisers on the physical and mechanical properties of films were higher for glycerol and smaller for sorbitol with xylitol demonstrating moderate effects. Thunwall *et al*. (2008) studied the effects of plasticisers on the properties of starch films. They reported that a glycerol-plasticised potato starch film produced by using hydroxypropylation/oxidation modified starch, had a significantly lower melt viscosity and a reduced shear-thinning behaviour compared with the native potato starch films, even at low plasticiser contents. They encountered difficulties in film processing due to

the sticky surface of the film, insufficient tenacity and foaming. They suggested that this behaviour was due to the presence of the high amount of glycerol. Thunwall *et al*. (2006b) reported that high amylose thermoplastic starch showed a higher melt viscosity than normal potato starch when conditioned at 53% RH. It was claimed that poor melt tenacity is one of the potential limitations when extruding thermoplastic starch at high temperatures. Water was reported to be restricting the higher processing temperature because vapour production in the material leads to bubbles and foaming, which are undesirable in film blowing. It was claimed that the high crystallinity in thermoplastic starch might be due to the hydrogen bonds formed between the starch and the plasticiser molecules. Godbillot *et al*. (2006) reported that the effect of glycerol on water vapour adsorption by plasticised wheat starch films depends on the equilibrium relative humidity and glycerol content. It was found in the study that below 44% equilibrium RH, glycerol plasticised wheat starch films were less hygroscopic than non-plasticised films. Above this percentage, phase separation occurred and the quantity of adsorbed water increased as it bound to the starch films and to the free glycerol. It was further reported that the hydration of plasticised starch films depends on the nature and amount of plasticiser and that water vapour adsorption is proportional to the number of hydrophilic sites in the plasticiser.

Mehyar and Han (2004) investigated the effect of glycerol (as a plasticiser) and RH on the physico-mechanical properties of high amylose rice starch and pea starch films. The tensile strength of both films decreased (3.2 to 1.9 high amylose rice starch, 4.2 to 2.8 MPa pea starch films) when the RH was increased from 51% to 90%, whereas the elongation at break of both films increased (265.6 to 751.4% high amylose rice starch, 323.7 to 420.9% pea starch films) with an increase in the RH. The study found that both films possessed good oxygen barrier properties and a high modulus of elasticity, although both films demonstrated

variable oxygen barrier properties at elevated RH. The WVP of both films were in the range of 130 to 150 g mm m^{-2} day^{-1} kPa^{-1}. The incorporation of glycerol into starch-based films, however, increased the solubility and moisture content in the films and also caused the high amylose granules to swell and continuously disperse between the amylopectin gels. Rodriguez *et al.* (2006) also studied the effects of glycerol, as a plasticiser, on the physical properties of starch-based films. They reported that glycerol interfered with starch packing, decreasing intermolecular attraction and increasing polymer mobility. Thus, glycerol caused a significant increase in the elongation at break (from 5.9 to 12.1%) of the film. They also reported that glycerol caused a decrease in the tensile strength of the starch-based films from 44.1 to 20.3MPa. In another study, Alves *et al.* (2007) investigated the effect of glycerol and amylose content on the barrier and mechanical properties of cassava starch films. They found that increasing the concentration of glycerol from 20 to 45% (w/w) in the film formulation resulted in a reduction in the tensile strength (from 21.7 to 5.4MPa). The study also demonstrated that increasing the glycerol level caused a decrease in the Young's modulus of the films. Incorporation of glycerol into cassava starch films, however, increased the elongation at break. The WVP of cassava starch films increased considerably from (2.4×10^{-9} to 4.9×10^{-9} g m^{-1} s^{-1} Pa^{-1}) when the concentration of glycerol in the film was raised from 20% to 45% (w/w).

Navarro-Tarazaga *et al.* (2008) studied the effect of the plasticisers glycerol and manitol on the mechanical properties of hydroxypropylmethylcellulose (HMPC)-beeswax coatings. They reported that an increase in glycerol and manitol concentrations increased the film flexibility. However, the WVP increased with the increase in glycerol concentration and remained unchanged with the change in manitol content. An increase in beeswax content reduced the film flexibility and the WVP of only glycerol-plasticised films. The effects of plastisers on

the properties of starch–MC blends plasticised with glycerol, sorbitol, xylose and water was studied by Arvanioyannis and Biliaderis (1999). These authors reported an increase in the WVP of starch-MC films and found it to be directly proportional to concentration of glycerol and sugars used to plasticise the film. Glycerol, sorbitol, xylose and water were observed to have lowered the tensile strength of starch-MC films. In contrast, glycerol, sorbitol, xylose and water increased the elongation at break of starch-MC films. Glycerol was observed to have a significant effect on the glass transition temperature in comparison to sorbitol.

Chaléat *et al.* (2008) investigated the effect of moisture content on the tensile and fracture properties of plasticised blended starch sheets prepared by compression moulding. The moisture content of extruded starch blends significantly affected the tensile properties of the blend due to changes in the glass transition temperature. Increasing the moisture content resulted in a reduction in the Young's modulus and yield stress. The study also showed that changes in moisture altered the fracture properties of the sheets. Higher moisture contents of the glycerol-plasticised sheets produced from thermoplastic potato starch lowered the stiffness of the compression-moulded starch-based materials as also pointed out by Thunwall *et al*. (2006a). They found that native high amylose starch exhibited a higher melt viscosity than modified starch. They also reported that the compression-moulded glycerol-plasticised high amylose starch had a higher elastic modulus and a higher tensile strength than the corresponding native starch. They also observed that the processing conditions during compression moulding had a great effect on the mechanical properties of thermoplastic starch. Gontard *et al.* (1993; 1996) studied the effect of the water content of wheat gluten films on the glass transition temperature and on the functional properties. They observed that this film behaved as an amorphous polymer. The glass transition temperature decreased with increasing water content. They reported a decrease in puncture resistance and in the modulus

of elasticity, but an increase in the water vapour transmission rate of the films at 5, 30 and 50°C. Rico-Peña and Torres (1991) determined the effects of water activity (a_w) and pH on the permeability constants of potassium sorbate and sorbic acid through MC-palmitic acid (PA) film. It was found in the study that the diffusion of sorbic acid decreased with an increase in pH. It was also found in the study that potassium sorbate diffusion rates in MC-PA film containing palmitic acid decreased with the decrease in a_w.

Sothornvit *et al.* (2007) investigated the effect of glycerol content on the mechanical properties of compression-moulded whey protein films. They found that increasing the glycerol content in the films from 30% (w/w) to 50% (w/w) decreased significantly the tensile properties of the films from 10 to 4 MPa. There was a reduction in Young's modulus when the glycerol content was increased. The study also demonstrated that changes in glycerol content increased the elongation at break of the films. McHugh and Krochta (1994) investigated the oxygen permeability and tensile properties of plasticised whey protein-based films. They observed that increasing the concentration of both glycerol and sorbitol resulted in a considerable reduction in the tensile strength and an increase in the percent elongation. Cuq *et al*. (1997) investigated the plasticising effect of sorbitol, glycerol and sucrose on the functional properties of fish myofibrillar protein-based films. Plasticisation of myofibrillar protein induced a significant decrease in film strength and elasticity, but increased the elongation at break and WVP. No significant differences were observed in the functional properties of myofibrillar protein-film when using glycerol, sorbitol and sucrose due to the structural similarities of these plasticisers. Chick and Ustunoi (1998) studied the effects of sorbitol and glycerol as plasticisers on the properties of lactic acid and rennet casein films and reported an increase in tensile strength of the films when the level of plasticiser was increased. They reported that sorbitol plasticised films were tougher than the glycerol

plasticised ones although glycerol plasticised films were more flexible. The percent elongation decreased with an increase in the protein-plasticiser ratio for the lactic acid casein films, whereas it increased for rennet casein films. Films plasticised with sorbitol were more effective moisture and oxygen barriers than the glycerol plasticised films. Wan *et al*. (2005) investigated the effects of various plasticisers including glycerol and sorbitol on the properties of soy protein isolate films. They reported that soy protein isolate films plasticised with sorbitol exhibited lower WVP values than the sorbitol-plasticised counterparts.

According to Forssell *et al.* (2002), starch-based films are sensitive to the surrounding RH, that may affect their barrier and mechanical properties when used in packaging of foods with a high water activity. Stading *et al.* (2001) studied the effect of the RH on the barrier, mechanical and thermal properties of amylose and amylopectin films. It was found that an increase in the RH of the surroundings leads to the plasticisation of amylose films. The RH during the formation of the glycerol-plasticised amylose films did not affect the crystallinity or glass transition temperature. Changong and Lumdubwong (2008) reported that RH had a significant effect on the mechanical properties of thermoplastic sheets extruded from starch-based materials. Olivas and Barbosa-Cánovas (2008) studied recently the effects of plasticiser and RH on the properties of alginate-calcium films. They found that increasing the RH decreases the tensile strength of these films. Conversely, an increase in the RH increases the elongation at break. A summary of the studies investigating the effects of plasticisers on the properties of protein-based films is presented in Table 2.2. Furthermore, some AM agents have demonstrated plasticising effects on packaging materials particularly in the case of biobased materials (Ahmad *et al.*, 2012). For example, citrus EOs incorporated into gelatine film demonstrated a plasticising effect (Tongnuanchan *et al.*, 2012). In their study, Türe *et*

al., (2012) indicated that potassium sorbate incorporated into wheat gluten films acts as plasticiser.

2.7.3 Effects of Lipids on the Properties of Biobased Films

The effects of lipids/fats on the properties of biobased films have been studied and reported in the scientific literature by different researchers (see Table 2.2). Permeability of AM films that are manufactured from biobased polymers is an essential consideration when preparing these films for food packaging. The biobased films and/or coatings can be used as barriers to moisture or gases such as oxygen that creates favourable conditions for microbial growth on food surfaces (Guilbert & Gontard, 2005; Rhim & Shellhammer, 2005). Films made from proteins and polysaccharide biopolymers demonstrate low gas permeability, but possess poor water barrier properties since they are hydrophilic in nature (Krochta *et al.*, 1994). However, the WVP of the films can be reduced by adding lipids to the film-forming solutions. Several studies explored the effects of adding oil to the film-forming solutions on the properties of AM films prepared from biobased films. For example, Garcia *et al.* (2000a) reported that the addition of sunflower oil to a starch-based film reduced the water vapour and gas permeability in comparison to starch-based films without oil. They found that the addition of oil to the film lowered the crystalline to amorphous ratio compared to films without oil additives. A study conducted by Yang and Paulson (2000), showed that the addition of lipids to gellan-based films enhanced significantly the WVP, although it deteriorated the mechanical properties and caused the films to become opaque. Shaw *et al.* (2002) studied the impact of varying the concentrations of soya oil and/or glycerol on the physical properties of whey protein isolate. It was found that increasing the concentration of oil led to an increase in percent elongation and glass transition temperature. The increase in oil concentration

however, lowered the moisture content, tensile strength and elastic modulus, but did not affect the WVP of the whey protein isolate films. An increase in the glycerol concentration led to an increase in percent elongation, moisture content, elastic modulus, glass transition temperature and opacity of the film.

Han *et al*. (2006) reported that the incorporation of beeswax into pea starch films affects the mechanical, physical and thermal properties of the films at high concentrations of beeswax. Incorporation of beeswax below the concentration of 30% was not successful in increasing the water resistance of the hydrophilic starch films. Beeswax addition decreased the WVP and increased the oxygen permeability. Chick and Hernandez (2002) studied the effect of carnauba and candelilla waxes on the properties of lactic acid casein-based films. They reported that an increase in wax content in these films decreased significantly the WVP. Shellhammer and Krochta (1997) studied the effects of beeswax, candelilla wax and milk fat on the WVP and mechanical properties of whey protein films. They reported that the addition of these additives decreased the tensile strength and Young's modulus of whey protein-lipid emulsion films. Möller *et al*. (2004) reported that chitosan-HMPC film incorporated with stearic acid exhibited a lower tensile strength (24 MPa) compared with stearic incorporated Chitosan-HMPC (30 MPa). The addition of stearic acid increased Young's modulus from 12 to 31 MPa of the chitosan-HMPC films. Conversely, the addition of stearic acid decreased the elongation at break of these films. Sebti *et al.* (2002) incorporated stearic acid into cellulose films and reported a decrease in water vapour transmission rate, increased contact angle, decreased tensile strength, and reduced air permeability of the films. Ozdemir and Floros (2003) found that increasing the concentration of beeswax in the film resulted in a decreased potassium sorbate diffusivity in whey protein films.

2.8 Antimicrobial Agents used in Synthetic Packaging Films

There are many different types of AM agents natural or synthetic that have the potential to be used to inhibit microbial growth in food products (Suppakul *et al.*, 2003b). Among the synthetic AM agents used are organic acids, fungicides, alcohols and antibiotics that possess a strong AM activity (Han, 2005a). However, the consumer preference for the use of natural AM agents such as basil, oregano and thyme EOs with their respective active constituents; linalool, carvacrol and thymol over synthetic AM agents has increased recently (Hammer *et al.*, 1999). In the past, chemical preservatives have been applied by dipping, surface spraying and direct incorporation into food products. However, the incorporation of EOs and their active constituents into packaging material is an emerging currently novel technique. When designing AM packaging systems containing volatile EOs and/or active constituents, many factors such as AM agent target (spoilage, pathogenic, broad spectrum, gram negative, gram positive, yeast and mould), release rate of the AM agent, water solubility and organoleptic properties need to be considered (Han, 2003; LaCoste *et al.*, 2005). Furthermore, storage and distribution conditions such as temperature are also put into consideration (Han, 2005a). In this subsection, a detailed summary of synthetic films utilising common synthetic and natural AM agents is presented with an emphasis on the principal components of basil, oregano, and thyme essential oils (EOs) namely, linalool, carvacrol and thymol respectively. This is followed by a list of other natural AM agents that have the potential for controlling microbial growth on foods. Final consideration is given to the organleptic properties of foods products as well as prospective applications of natural AM agents.

2.9 Incorporation of Volatile Agents into Films and Their Storage

In AM systems, volatile AM agents can be incorporated directly into a polymer during processing. Nonetheless, the method of incorporation of volatile AM agents into the packaging is one of the imperative factors that have significant effect on the resultant films. During extrusion or compression moulding of AM films, the temperature and mechanical energy input, such as shearing forces, must be carefully considered (Han, 2003). High-processing temperatures, for example, may result in considerable losses of volatile AM agents (Han, 2000; Han & Floros, 1997; Rupika *et al.*, 2005; Suppakul *et al.*, 2011a). Moreover, Cooksey (2005) suggested that an AM agent might partly or completely loose its AM activity if incorporated into a film under harsh processing conditions. Therefore, to minimise the loss of AM agent during processing, temperatures that are as low as possible should be applied (Han & Floros, 1998a; Suppakul *et al.*, 2011a).

The storage temperature may also influence the activity of AM agents that are incorporated into packaging films (Han, 2005a; Vojdani & Torres, 1989). The concentration of AM agents retained in the film may decrease during long-term storage. However, the amount of AM agent retained in the film after a long storage period may be sufficient to demonstrate AM activity as shown by Suppakul *et al.* (2011a). Du *et al.* (2008b) reported AM activity against *E. coli* (using an agar disc diffusion method) of carvacrol incorporated into films for edible apples that were stored for 7 weeks.

2.10 The Release of Volatile Agents Incorporated into Packaging Films

The mode of action of AM agents incorporated in a packaging material is by the controlled release technology where the agent is slowly released onto the food surfaces in order to

maintain an adequate concentration of the agent on the food (Cooksey, 2005; Marlene *et al.*, 2010; Salleh *et al.*, 2007; Tunç & Duman, 2011). The release rate of AM agents from the packaging material has a significant influence on the AM activity and potential applications of AM films in food packaging (Chung *et al.*, 2001b; LaCoste *et al.*, 2005; Rardniyom *et al.*, 2008b). An AM agent incorporated into a packaging material is released mainly by permeation and diffusion onto food surfaces to control pathogenic and/or spoilage microorganisms during the storage period (Buonocore *et al.*, 2003a; Chen *et al.*, 1996; Han & Floros, 1998b; Limm & Holifield, 1995; López-Rubio *et al.*, 2004; Ozdemir & Floros, 2003; Quintavalla & Vicini, 2002). Han, (2005a) suggested that the mass transfer rate of an AM agent should not be faster than the growth rate of the target microorganism, otherwise the AM agent might be diluted on the surface of the packaged food product, thus limiting the AM activity. Conversely, when the release rate is too slow to control the growth of the microorganisms; the microorganisms may grow faster then the AM agent is released. Thus, the release rate of AM agents from the packaging material onto food should be specifically controlled to be similar to the growth rate of the target microorganisms (Han, 2000).

The release rate of the AM agent from the packaging material is primarily influenced by factors that include the film fabrication method, the properties of the AM agent (such as volatility and polarity), the chemical interaction between the AM agent and polymer chains, changes in the packaging film that might be induced by the AM agent incorporated into the film, properties such as hydrophobicity and hydrophilicity of the polymer, food composition, properties such as water activity (a_w) and pH of the food, and environmental factors such as storage conditions, primarily temperature and relative humidity (Suppakul *et al.*, 2003b; Weng & Hotchkiss, 1993). In most cases, it is complicated, time consuming and expensive to determine the migration of AM agent into the food because most foodstuffs are comprised of

a complex mixture of substances such as water, carbohydrates, fats, lipids, proteins, vitamins, fibres and minerals (Marlene *et al.*, 2010). Thus, migration studies are usually performed using food simulants (Dopico *et al.*, 2003). Different food simulants have been suggested in the European food-packaging regulations (EC, 1997) for migration testing. The food simulants for various food products include: water (for water-based products); 3% (v/v) acetic acid in water (for acidic products); 50% (v/v) ethanol in water (for dairy products); olive oil; sunflower oil; and synthetic fat simulant HB 307; 95% ethanol in water and isooctane for fatty products (EC, 1997; FDA, 2007). Very recently, new simulants have been recommended by the EC (Regulation 10/2011) for different food products. This regulation is aimed to be implemented gradually and will become compulsory from January 1, 2016 (EC, 2011). The compatibility of an AM agent with different types of foods or food simulants is an important factor that must be considered when designing AM packaging systems (Rardniyom *et al.*, 2008b).

2.11 Antimicrobial Activity of Antimicrobial Agents

2.11.1 Synthetic AM Agents

Many synthetic AM compounds have been evaluated in synthetic polymers by various researchers. Table 2.3 summarises these synthetic AM agents incorporated into or coated onto packaging materials as potential candidates for food packaging. Although Table 2.3 contains a large amount of information on the activity of AM agents successfully incorporated into various synthetic polymers, comparison between the different AM agents and/or AM films is difficult due to variations in strains of microorganisms and different experimental conditions or equipment used by the various researchers. In order to compare

the results of various experiments involving AM agents, there is a need for a standardisation of the test methods as suggested by Suppakul *et al.* (2003b).

Table 2.3 Antimicrobial activity of common synthetic AM agents

AM Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
Benzoic acid	0.5 mol/L	PEMA	PDA	*A. niger* and *Penicillium* sp.	Inhibited microbial growth	Weng *et al.* (1999)
Benzoic acid	0.5-2% w/w	LDPE	Agar media; Cheddar cheese	*R. stolonifer*, *Penicillium* sp. and *A. toxacarius*	Failed to inhibit mould growth	Weng & Hotchkiss (1993)
Benzoic anhydride	0.5-2% w/w	LDPE	Agar media; Cheddar cheese	*R. stolonifer*, *Penicillium* sp. and *A. toxacarius*	Demonstrated antimycotic activity on media and cheese	Weng & Hotchkiss (1993)
EDTA	5% w/w	LDPE	Agar diffusion	*B. subtilis*, *A. niger* and *E. coli*	Inhibited *B. subtilise* and *A. Niger* but not *E. coli*	Vartiainen *et al.* (2003)
Imazalil	1000-2000 mg/kg	LDPE	Agar media; Cheddar cheese	*A. toxacarius*, *Penicillium* sp.	All concentrations delayed microbial growth on media and cheese	Weng & Hotchkiss (1992)
Imazalil	0.05-0.25% w/w	LDPE	Agar diffusion	*B. subtilis*, *A. niger* and *E. coli*	Inhibited *B. subtilise* and *A. Niger* but not *E. coli*	Vartiainen *et al.* (2003)
Potassium sorbate	2-3% w/v	PVC	Agar media	*L. monocytogenes*	Films inhibited microbial growth	Limjaroen *et al.* (2003)
Propionic acid	0.5-2% w/w	LDPE	Agar media;	*R. stolonifer*, *Penicillium* sp.	Failed to inhibit mould growth	Weng & Hotchkiss (1993)

AM Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
			Cheddar cheese	and *A. toxacarius*		
Propionic anhydride	0.5-2% w/w	LDPE	Agar media; Cheddar cheese	*R. stolonifer*, *Penicillium* sp. and *A. toxacarius*	Failed to inhibit mould	Weng & Hotchkiss (1993)
Sodium propionate	0.5-2% w/w	LDPE	Agar media; Cheddar cheese	*R. stolonifer*, *Penicillium* sp. and *A. toxacarius*	Failed to inhibit mould	Weng & Hotchkiss (1993)
Sodium diacetate	0.5-3% w/v	PVC	Agar media	*L. monocytogenes*	No AM activity observed	Limjaroen *et al.* (2003)
Sorbic acid	1.5-3% w/v	PVC	Agar media	*L. monocytogenes*	Inhibited microbial growth	Limjaroen *et al.* (2003)
Sorbic acid	0.5-2% w/w	LDPE	Agar media; Cheddar cheese	*R. stolonifer*, *Penicillium* sp. and *A. toxacarius*	Failed to inhibit mould	Weng & Hotchkiss (1993)
Sorbic acid	0.5 mol L^{-1}	PEMA	PDA	*A. niger* and *Penicillium* sp.	Inhibited microbial growth	Weng *et al.* (1999)
Triclosan	500-1000 mg kg^{-1}	LDPE	Agar diffusion; Chicken breasts	*L. monocytogenes, Sal. enteritidis, Staph. aureus, E. coli* O157:H7, *B. thermosphacta, B. cereus, L. sake, L. brevis, P. Roqueforti, A.*	Inhibited *L. monocytogenes, Sal. enteritidis, Staph. aureus, E. coli* O157:H7 with slight inhibition of *B. thermosphacta*, but no activity against, *B.*	Vermeiren *et al.* (2002)

AM Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
				niger and *C. albicans*	*cereus, L. sake, L. brevis, P. Roqueforti, A. niger* and *C. Albicans*	
Triclosan	5% w/w	PVC	Plate count	*Staph. aureus, E. coli*	*Staph. aureus, E. coli*	Ji and Zhang (Ji & Zhang, 2009)

Numerous studies have concentrated on incorporating common food preservatives such as organic acids, their salts and anhydrides into packaging films (see Table 2.3). Studies on benzoic or sorbic acid incorporated into packaging materials have evaluated their action against various microorganisms in laboratory media such as agar plates and/or in actual food products. The packaging films incorporated with these organic acids or anhydrides have demonstrated inhibitory effects against various spoilage and pathogenic microorganisms. Weng *et al.* (1999) showed that benzoic or sorbic acids incorporated into poly(ethylene-co-methacrylic acid) (PEMA) film inhibited the growth of *A. niger* and *Penicillium* sp. on solid media. Weng and Chen (1997) investigated the AM activity of benzoic acid or benzoyl chloride incorporated into ionomer films. The AM activity of these films was demonstrated by their ability to inhibit the growth of *Penicillium* sp. and *A. niger*. In an earlier study, Weng and Hotchkiss (1993) incorporated benzoic acid or benzoic anhydride into LDPE films which significantly suppressed the growth of *Rhizopus stolonifer*, *Penicillium* sp. and *A. toxacarius* on potato dextrose agar and on the surface of Cheddar cheese. Matche *et al.* (2006) examined the AM activity of benzoyl chloride incorporated into modified ethylene acrylic acid films against *Penicillum* sp. and *A. niger* sp. on solid media for 15 days with the film demonstrating inhibition against both species. Silveira *et al.* (2007) incorporated sorbic acid into LDPE films with the aim of preserving fresh pastry dough. It was found that 3% (w/w) sorbic acid incorporated into a 70 μm film reduced 2 and 1.5 log cycles of mesophilic and psychrotrophic bacteria respectively on the pastry dough after 40 days of storage at 8°C compared to the control film. Limjaroen *et al.* (2005) coated sorbic acid onto polyvinylidene chloride copolymer films to control the growth of *L. monocytogenes* on beef bologna and Cheddar cheese. It was found that sorbic acid coated on the films inhibited microbial growth on cheese by log 0.6 $CFUg^{-1}$ after 28 days of storage at 4°C. They further reported that the

population of *L. monocytogenes* on beef bologna was reduced by log 0.6 and 1.4 $CFUg^{-1}$ for films containing 1.5% and 3.0% (w/v) respectively compared to the control film.

Other researchers have studied the AM activity of salts against several microorganisms as shown in Table 2.3. Han and Flores (1997) developed LDPE films containing potassium sorbate and found that these films successfully reduced the growth of *S. cerevisiae in vitro* experiments. Vartiainen and others (2003b) demonstrated that potassium sorbate, sodium benzoate and sodium nitrate incorporated into LDPE, poly(maleic acid-co-olefine), PET or PS films inhibited the growth of *B. cereus* on culture media. Limjaroen and others (2003) coated potassium sorbate onto polyvinylidene chloride copolymer films and reported that the films inhibited the growth of *L. monocytogenes* on solid media.

In addition to the antibacterial activity, also the antifungal activity of synthetic AM agents incorporated in polymeric materials has been investigated (Halek & Anita, 1989; López-Malo *et al.*, 2007). Vartiainen and others (2003a) examined the inhibitory effects of imazalil incorporated into LDPE against the growth of *A. niger* by the agar diffusion assay with films containing 0.05-0.25% (w/w) imazalil demonstrating significant inhibitory activity. Weng and Hotchkiss (1992) incorporated imazalil into LDPE film and evaluated the antimycotic activity of this agent on the growth of *A. toxacarius* and *Penicillium* sp. on potato dextrose agar (PDA) and Cheddar cheese. They reported that 2 g kg^{-1} of imazalil suppressed the growth of *A. toxacarius* on PDA whereas a film containing 1 g kg^{-1} of imazalil reduced the growth of *Penicillium* sp. The latter film inhibited the growth of both mould species on the surface of Cheddar cheese. López-Malo *et al.* (2002; 2005) examined the antifungal activity of potassium sorbate, sodium benzoate and sodium bisulfite against the growth of *Aspergillus flavus* inoculated on laboratory media with each of the agents imparting an inhibitory effect.

López-Malo *et al.* (2007) investigated the antifungal activity of sodium benzoate and cinnamon extract, separately or in combination, against the growth of *A. flavus* on potato dextrose agar or a checkerboard array respectively. They found that both AM agents demonstrated antifungal activity on *A. flavus*, with cinnamon extract being more effective than sodium benzoate. They claimed that mixtures of cinnamon extract and sodium benzoate showed promising antifungal activity.

Triclosan and hexamethylenetetramine are common synthetic AM agents that have been evaluated in packaging systems with some commercial developments. Vermeiren *et al.* (2002) investigated the AM activity of triclosan incorporated into LDPE film and reported that concentrations of 0.5 and 1.0% (w/w) demonstrated AM activity against *L. monocytogenes, Sal. enteritidis, Staph. aureus, E. coli* O157:H7 and *Brocothrix thermosphacta* in an agar diffusion assay. Cutter (1999) reported that triclosan was effective against bacteria on the surface of beef. Recently, Camilloto *et al.* (2010) studied the activity of triclosan incorporated into LDPE against *Staph. aureus*, *E. coli*, *L. innocua* and *P. aeruginosa* in the agar disc diffusion test and found that the AM film inhibited the growth of *Staph. aureus* and *E. coli*. Chung *et al.* (2003b) investigated the AM activity of triclosan coated onto a styrene-acrylate copolymer against *Enterococcus faecalis* on solid and in liquid media and showed effective inhibition of the bacteria. Ji and Zhang (2009) reported that triclosan incorporated into PVC film inhibited the growth of *Staph. aureus* and *E. coli* using the plate-counting technique. Devlieghere *et al.* (2000c) studied the AM activity of hexamethylenetetramine impregnated into an LDPE film and found it to be effective against spoilage microorganisms on cooked ham.

2.11.2 Natural Antimicrobial Agents

Table 2.4 summarises the AM activity of a range of common natural agents that have been incorporated into or coated onto synthetic packaging films. Table 2.4 also lists other studies that have evaluated the inhibitory effects of natural AM agents *in vitro* or directly on food products without incorporating them into packaging films.

Antimicrobial Activity of Basil Essential Oils

Basil EOs contain primarily linalool and methylchavicol as the active volatile components which are responsible for their AM activity (Bezic *et al.*, 2003; Fyfe *et al.*, 1998; Simon *et al.*, 1990; Suppakul *et al.*, 2003b; Wan *et al.*, 1998). Many studies have evaluated the AM activity of basil EOs against various microorganisms both *in vitro* and on a range of food products as shown in Table 2.4. Prasad *et al.* (1986) investigated the AM activity of the EOs of *O. basilicum* against various Gram-positive and Gram-negative bacteria with the oils shown to be more effective against Gram-positive bacteria including *Bacillus sacharolyticus, B. stearothermophilus, B. subtilis, B. thurengiensis, Micrococcus glutamicus* and *Sarcina lutea* than the Gram-negative ones. Lachowicz *et al.* (1998) evaluated the AM effects of EOs of sweet basil against acid-tolerant food microflora. They reported greater inhibitory effects of the tested EOs against the Gram-positive bacteria *Bacillus* sp., *Staph. aureus* sp., *Micrococcus* sp., *Sarcina* sp., *Lactobacillus* sp. than against the Gram-negative ones *E. coli, Salmonella* sp., *Enterobacter* sp. and *Pseudomonas* sp. In contrast to these studies, Koga *et al.* (1999) found that the Gram-positive bacteria were more resistant to basil EOs than the Gram-negative ones.

Table 2.4 Antimicrobial activity of natural AM agents

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
Carvacrol	1.33-2.65% w/w	LDPE	Liquid culture	*E. coli*	Reduced microbial growth	Rupika *et al.* (2008b)
Carvacrol	1-4% w/w	PP	Agar medium	*E. coli, Y. enterocolitica, P. aeruginosa, Staph. aureus, B. cereus* and *E. faecalis, C. albicans, D. hansenii, Z. Rouxii, A. flavus, E. repens, p. roqueforti, P. commune*	Carvacrol demonstrated AM activity against all the tested microorganisms	Gutierez *et al.* (2009b)
Carvacrol	1-4% w/w 1-21.8µL/L	PP, PE/EVOH	Agar medium or vapour diffusion method	*L. monocytogenes, Sal. choleraesuis, A. flavus* and *C. albicans*	carvacrol inhibited the growth of all the tested microorganisms	Lopez *et al.* (2007a; 2007b)
Carvacrol	0.2-2% w/w	LDPE	Agar media	*E. coli, Staph. aureus, L. innocua, P. aeruginosa, A. niger* and *S. cerevisiae*	Inhibited *E. coli, Staph. aureus, A. niger* and *S. cerevisiae* but not *L. innocua* or *P. aeruginosa*	Rupika *et al.* (2005)
Carvacrol	10% w/w	LDPE	Agar media	*B. thermosphacta, L. innocua* and *Carnobacterium. sp*	Carvacrol demonstrates *B. thermosphacta, L. innocua* and *Carnobacterium. sp*	Persico *et al.* (2009)

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
Carvacrol	4% w/w	LDPE and nylon film	liquid food media; Cheddar cheese	*E. coli*	Multilayer films inhibited microbial growth	Rardniyom *et al.* (2008b)
Cinnamaldehyde	1-4% w/w	PP	Agar medium	*E. coli, Y. enterocolitica, P. aeruginosa, Staph. aureus, B. cereus* and *E. faecalis, C. albicans, D. hansenii, Z. Rouxii, A. flavus, E. repens, p. roqueforti, P. commune*	cinnamaldehyde demonstrated AM activity against all the tested microorganisms	Gutiérrez *et al.* (2009b)
Cinnamaldehyde	1-4% w/w 0.4-21.8µL/L	PP, PE/EVOH	Agar medium or vapour diffusion method	*L. monocytogenes, Sal. choleraesuis, A. flavus* and *C. albicans*	Cinnamaldehyde inhibited the growth of all the tested microorganisms	Lopez *et al.* (2007a)
Clove extract	20% w/w	LDPE	Liquid culture	*E. coli, L. plantarum, S. cerevisiae* and *F. oxysporum*	Effective against *L. plantarum* and *F. oxysporum* but not against *E. coli* and *S. cerevisiae*	Hong *et al.* (2000)
GFSE	0.1 or 1% w/w	LDPE	Agar media; Curled lettuce;	*E. coli, Staph. aureus, L. mesenteroides, S. cerevisiae, A.*	Inhibited *E. coli* and *Staph. aureus* but not *S. cerevisiae, A.*	Lee *et al.* (1998)

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
			Soybean sprouts	*oryzaei*, *A. niger*, *P. chrysogenum*	*oryzaei*, *A. niger*, or *P. chrysogenum*.	
GFSE	0.5% or 1% w/v	Multi-layered PE (coated)	Ground beef, Agar media	*M. flavus*, *P. aeruginosa*, *E. coli*, *Staph. aureus* and *B. subtilise*, *S. cerevisiae*, *A. Niger*, *P. chysogenum*, *L. mesenteroides*	AM activity against *M. flavus*, *E. coli*, *Staph. aureus* and *B. subtilise*	Ha *et al.* (2001)
Lactoferrin	0.5-2.5% w/v	PVC	Agar media	*L. monocytogenes*	No AM activity	Limjaroen *et al.* (2003)
Lacticin NK24	20 gL^{-1}	LDPE	Fresh oysters; ground beef	Coliform, total aerobic bacteria	Inhibited microbial growth	Kim *et al.* (2002c)
Linalool	0.037% w/w	LDPE	Agar media	*E. coli*	Linalool incorporated into LDPE film inhibit the growth of *E. Coli* after 1 year of storage	Suppakul *et al.* (2011a)
Linalool	0.338% w/w	LDPE	Agar media; Cheddar cheese	*E. coli*, *L. innocua*, *S. cerevisiae*	Inhibitory activity against *E. Coli* but not *L. innocua* or *S. cerevisiae* on agar media; reduced *E. coli* and *L. innocua* on cheese	Suppakul *et al.* (2004; 2006; 2008)
Linalool	4% w/w	LDPE and nylon film	Liquid culture; Cheddar cheese	*E. coli*	Multilayer films inhibited microbial growth	Rardniyom *et al.* (2008b)
Linalool	20µL	Added into cellulose disc	Solid media	Various bacteria including *E. coli*, *Staph. aureus*, *seven*	Inhibited microbial growth	Mazzanti *et al.* (1998)

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
				strains of Candida		
Linalool	0.54-1.19% w/w	LDPE	Agar and liquid media; Cheddar cheese	*E. coli, L. innocua*	Inhibited microbial growth	Rupika *et al.* (2006)
Linalool	15µL	Direct application	Solid media	25 strains of bacteria	Inhibited microbial growth	Dorman & Deans (2000)
Methylchavicol	0.028% w/w	LDPE	Agar media	*E. coli*	Methylchavicol inhibitory activity against the growth of *E. Coli* after 1 year of storage	Suppakul *et al.* (2011a)
Methylchavicol	0.345% w/w	LDPE	Agar media; Cheddar cheese	*E. coli, L. innocua, S. cerevisiae*	Inhibitory activity against *E. coli* but not against *L. innocua* or *S. cerevisiae* on agar media	Suppakul *et al.* (2004; 2006; 2008)
Nisin	0.05 or 0.1% w/v	LDPE	Beef carcass	*B. thermosphacta*	Inhibited microbial growth	Siragusa *et al.* (1999)
Nisin	2-2.5% w/v	PVC	Agar media	*L. monocytogenes*	Inhibited microbial growth	Limjaroen *et al.* (2003)
Nisin	157 mg/mL	LDPE	Agar media	*L. monocytogenes*	Inhibited microbial growth	Grower *et al.* (2004)
Nisin	100 µg/mL	LLDPE, PVC, nylon	Broiler skin	*Sal. typhimurium*	Significantly reduced microbial population	Natrajan & Sheldon (2000b)
Nisin	20 g/L	LDPE	Fresh oysters; ground beef	coliform, total aerobic bacteria	Suppressed coliform and bacterial growth	Kim *et al.* (2002c)
Nisin	0.03 or 0.6	PE or	Sliced cheese;	*L. innocua* and *Staph. aureus*	Reduced microbial growth in	Scannell *et al.* (2000)

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
	g/mL	polyamide	ham		cheese	
Propolis	20% w/w	LDPE	Liquid culture	*E. coli*, *L. plantarum*, *S. cerevisiae* and *F. oxysporum*	Inhibited *L. plantarum* and *F. oxysporum* but not *E. coli* or *S. cerevisiae*	Hong *et al.* (2000)
Thymol	0.85-3.15% w/w	LDPE	Liquid culture	*E. coli*	Inhibited microbial growth	Rupika *et al.* (2008b)
Thymol	1-4% w/w	PP	Agar medium	*E. coli*, *Y. enterocolitica*, *P. aeruginosa*, *Staph. aureus*, *B. cereus* and *E. faecalis*, *C. albicans*, *D. hansenii*, *Z. Rouxii*, *A. flavus*, *E. repens*, *p. roqueforti*, *P. commune*	Thymol demonstrated AM activity against all the tested microorganisms	Gutiérrez *et al.* (2009b)
Thymol	0.23-1.6% w/w	LDPE	Agar media	*E. coli*, *Staph. aureus*, *L. innocua*, *P. aeruginosa*, *A. niger* and *S. cerevisiae*	Inhibited *E. coli*, *Staph. aureus*, *A. niger* and *S. cerevisiae* but not *L. innocua*, and *P. aeruginosa*	Rupika *et al.* (2005)
Thymol	1-4% w/w 1-21.8µL/L	PP, PE/EVOH	Agar medium or vapour diffusion method	*L. monocytogenes*, *Sal. choleraesuis*, *A. flavus* and *C. albicans*	Thymol inhibited the growth of all the tested microorganisms	Lopez *et al.* (2007a)
Basil Eos	10µL of undiluted oils	Direct application	Solid media	25 strains of test organisms	Inhibited microbial growth	Baratta *et al.* (1998)
Basil EOs	0.01-1% (v/v)	Direct	Solid media	Against wide range of	Inhibitory effects against Gram-	Lachowicz *et al.* (1998)

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
		application	Tomato juice	microorganisms including *Bacillus* sp., *Staph. aureus* sp., *micrococcus* sp., *Sarcina* sp., *Lactobacillus* sp., *E. coli*, *Salmonella*, sp., *Enterobacter* sp., *Pseudomonas* sp.	positives (*Bacillus* sp., *Staph. aureus* sp., *micrococcus* sp., *Sarcina* sp. and *Lactobacillus* sp. Reduced effects against Gram-negatives (*E. coli*, *Salmonella* sp., *Enterobacter* sp. and *Pseudomonas* sp.	
Basil EOs	500-3.9µL/mL oil dilution	EOs added into paper disc	Solid medium	*Fusarium acuminatum, F. solani, F. pallidoroseum* and *F. chlamydosporum*	EOs effective against all *Fusarium* species	Rai *et al.* (1999)
Cinnamon EOs	1-4% w/w 13.1-131µL/L	PP, PE/EVOH	Agar medium or vapour diffusion method	*E. coli, Y. enterocolitica, P. aeruginosa, Staph. aureus, B. cereus* and *E. faecalis, C. albicans, D. hansenii, Z. Rouxii, A. flavus, E. repens, p. roqueforti, P. commune*	Cinnamon EOs in PP or PE/EVOH demonstrated AM activity against all the tested microorganisms	Lopez *et al.* (2007a; 2007b)
Cinnamon EOs	3-6% (w/w)	Paraffin-based paper	*In vitro*, Sliced bread	*A. alternata*	Cinnamon EOs inhibited the growth of *A. alternata* on solid media	Rodriguez-Lafuente *et al.* (2010)
Cinnamon EOs	1-6% w/w	Paraffin-paper	*In vitro*, Sliced bread	*R. stolonifer*	Cinnamon EOs in paraffin film inhibited the growth of *R.*	Rodríguez *et al.* (2008)

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
					stolonifer	
Clove	1-4% w/w	PP, PE/EVOH	Agar medium	*E. coli, Y. enterocolitica, P. aeruginosa, Staph. aureus, B. cereus* and *E. faecalis, C. albicans, D. hansenii, Z. Rouxii, A. flavus, E. repens, p. roqueforti, P. commune*	Clove EOs demonstrated AM activity against all the tested microorganisms	Lopez *et al.* (2007a; 2007b)
Oregano EOs	0.8% (v/w)	surface dipping, O_2 permeable films	beef meat fillets	*L. monocytogenes,* autochthonous flora	Reduced growth by 2-3 $\log_{10}$	Tsigarida *et al.* (2000)
Oregano EOs	3-6% (w/w)	Paraffin-based paper	*In vitro,* Cherry tomato	*A. alternata*	Oregano EOs inhibited the growth of *A. alternata* on solid media	Rodriguez-Lafuente *et al.* (2010)
Oregano EOs	1-2 g	dressing, MAP	fresh fish fillets	*Staph. aureus, Sal. enteritidis,* Residential flora	Bacterostatic and bactericidal effects	Tassou *et al.* (1996)
Oregano EOs	800 ppm	surface spreading	thin-sliced beef	*L. monocytogenes*	Significant inhibition	Seaberg *et al.* (2003)
Oregano EOs	0.05-1% (v/w)	PE bags	minced beef	spoilage microbiota	Reduction in microbial loads	Skandamis and Nychas (2001)
Oregano EOs	0.05% (v/w)	surface application	cod fish fillets	*Photobacterium phosphoreum*	No significant growth	Mejlholm and Dalgaard (2002)
Oregano EOs	1-4% w/w	PP, PE/EVOH	Agar medium or	*E. coli, Y. enterocolitica, P.*	Oregano EOs in PP or	Lopez *et al.* (2007a;

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
	13.1-175µL/L		Vapour diffusion method	*aeruginosa, Staph. aureus, B. cereus* and *E. faecalis, C. albicans, D. hansenii, Z. Rouxii, A. flavus, E. repens, p. roqueforti, P. commune*	PE/EVOH demonstrated AM activity against all the tested microorganisms	2007b)
Oregano EOs	50 µL	Suspensions of oils in apple juice	Apple juices	*E. coli, Sal. enterica*	Selected oils were bactericidal	Friedman *et al.* (2004b)
Oregano EOs	0.1-10% (v/v)	dissolved in brain heart infusion broth	Liquid culture	*Sal. enterica*	OEO showed strongest AM activity	Marques *et al.* (2008)
Oregano EOs			*In vitro*	*B. cereus, E. coli L. monocytogenes*	Inhibited microbial growth	Baydar *et al.* (2004)
Thyme EOs	50 µL	vapour contact	sponge cake analogues	*Eurotium* sp., *Aspergillus* sp., *Pencillium* sp.	Significant reduction in microbial growth	Guynot *et al.* (2003)
Thyme EOs	135 or 270 µL/L	vapour contact	rye bread	*Pencillium* sp., *E. repens, A. flavus*	Significant reduction in microbial growth	Suhr and Nielsen (2003)
Thyme EOs	0.1-1% (v/v)	cheese-EO-mixture	soft cheese	*L. monocytogenes* *Sal. enteritidis*	Significant inhibition in low-fat cheese; no inhibition in full-fat cheese	Smith-Palmer *et al.* (2001)
Thyme EOs	1:5 dilution	surface application	cooked poultry	*A. hydrophila, L. monocytogenes*	Inhibited growth of *A. Hydrophila*	Hao *et al.* (1998)
Thyme EOs	1-4% w/w	PP, PE/EVOH	Agar medium,	*E. coli, Y. enterocolitica, P.*	thyme EOs demonstrated AM	Lopez *et al.* (2007a;

Antimicrobial Agent	Amount added	Packaging Material	Test Type/Media	Target Microorganism(s)	Findings	References
	26.2-175µL/L		Vapour diffusion method	*aeruginosa, Staph. aureus, B. cereus* and *E. faecalis, C. albicans, D. hansenii, Z. Rouxii, A. flavus, E. repens, p. roqueforti, P. commune*	activity against all the tested microorganisms	2007b)
Thyme EOs		surface application	raw fish fillets	*Photobacterium phosphoreum*	No significant growth reduction	Mejlholm and Dalgaard (2002)

Various researchers have reported also the inhibitory effect of basil EOs against fungi. Rai *et al.* (1999) evaluated the antifungal activity of the EOs of ten plant species (including *O. basilicum*) and reported that the EOs of basil were active against all *Fusarium* species including *F. acuminatum, F. solani, F. pallidoroseum* and *F. chlamydosporum.* Conner and Beuchat (1984) reported positive AM activity of basil EOs against *Kloeckera apiculata* on solid media. Basilico and Basilico (1999) investigated the inhibitory effects of some EOs, including that of basil (*O. basilicum*), against the growth of *A. ochraceus* and subsequent ochratoxin A production. They reported that at a level of 1000 ppm, only basil EO decreased the fungal growth and the production of ochratoxin A for up to 7 days after which mould growth occurred.

Antimicrobial Activity of Linalool

Linalool has been reported to possess both fungistatic and antibacterial properties against a wide spectrum of microorganisms such as *Staph. aureus, L. innocua, E. coli, A. niger* and *S. cerevisiae* (Friedman *et al.*, 2002; Lachowicz *et al.*, 1998; Suppakul *et al.*, 2003b). As shown in Table 2.4, numerous studies have evaluated the AM activity of linalool incorporated into packaging films. For example, Suppakul *et al.* (2006; 2008) reported that linalool incorporated into LDPE film exhibited inhibitory activity against the growth of *Staph. aureus, L. innocua, E. coli* and *S. cerevisiae* on culture media and on the surface of Cheddar cheese. Rardniyom (2008a) investigated the AM activity of linalool coated onto LDPE and nylon films against the growth of *E. coli* and reported effective inhibitory activity in liquid culture and on Cheddar cheese. Rupika *et al.* (2006) reported that linalool incorporated into LDPE films demonstrated significant inhibitory activity against the growth of *L. innocua* and *E. coli* both *in vitro* and on the

surface of Cheddar cheese. Suppakul *et al.* (2011a) reported that linalool and/or methylchavicol incorporated into LDPE films demonstrated inhibitory activity against the growth of *E. coli* on agar disc media.

The AM activity of linalool against several microorganisms has also been reported in studies conducted *in vitro* only. Kim *et al.* (1995a) investigated the AM activity of some EO components including linalool against five food-borne pathogens (*E. coli, E. coli* O157*: H7, Sal. typhimurium, L. monocytogenes and V. vulnificus*) and found a dose-related increase in the zone of inhibition against all tested strains except for *L. monocytogenes*. Mazzanti *et al.* (1998) reported that linalool completely inhibited the growth of all yeasts (seven strains of *C. albicans*, *C. krusei* and *C. tropicalis*), *Staph. aureus* and *E. coli* using the agar disc diffusion method. Dorman and Deans (2000) investigated the antibacterial activity of 21 plant volatile oil components including linalool against 25 bacterial strains using the agar well diffusion method. It was reported in this study that linalool was an effective AM agent against a broad spectrum of 23 out of the 25 bacterial strains investigated.

Antimicrobial Activity of Oregano and Thyme Essential Oils

Oregano and thyme are popular culinary herbs with their EOs containing terpenoid compounds, mainly the monoterpenoid phenols of thymol (5-methyl-2-[1-methylethyl] phenol) and carvacrol (5-isopropyl-2-methyl phenol). These EOs have been claimed to demonstrate potential health benefits, antioxidant activity and AM properties (Baratta *et al.*, 1998; Davidson & Taylor, 2007; Nychas, 1995; Olasupo *et al.*, 2004; Tepe *et al.*, 2004; Youdim & Deans, 2000). The AM activity of thyme and oregano EOs is

primarily attributed to their major components thymol and carvacrol respectively (Bagamboula *et al.*, 2004; Cosentino *et al.*, 1999; Davidson & Naidu, 2000; Davidson & Taylor, 2007; Dorman & Deans, 2000; Farag *et al.*, 1989; Lambert *et al.*, 2001).

The AM activity of oregano and thyme EOs against various microorganisms has been investigated on media and on a range of food products as summarised in Table 2. For example, Lin *et al.* (2004) evaluated the AM activity of phenolic compounds derived from oregano against *L. monocytogenes* on solid media, on beef and on fish products and reported that the extracts exhibited AM activity against *L. monocytogenes* in the agar diffusion assays. Friedman (2004b) studied the antibacterial activity of ten different EOs including that of oregano against *E. coli* and *Sal. enterica* in apple juice. They reported that the selected EOs exhibited greater AM activity against *Sal. enterica* than *E. coli*.

The AM activity of oregano and thyme EOs was investigated in liquid culture and solid media against various microorganisms. Becerril *et al.* (2007) investigated the AM activity of oregano EOs incorporated into a patented plastic packaging material against *E. coli* and *Staph. aureus*, using a "kill time" assay. The authors claimed that oregano EOs exhibited significant AM activity with a “kill time” of approximately 90 min for *E. coli* and 104 min for *Staph. aureus*. Marques *et al.* (2008) studied the AM activity of three natural AM agents: oregano, garlic and chitosan against the growth of *Sal. enterica* in liquid culture at 10 and 20°C. They reported that all of the natural agents inhibited significantly the microbial growth at both temperatures with oregano demonstrating the highest inhibitory effects followed by garlic then chitosan.

Rodriguez-Lafuente *et al.* (2010) investigated the AM activity of oregano and cinnamon EOs incorporated into packaging-paper against *Alternaria alternata* using an *in vitro* antifungal assay. The authors reported that oregano and cinnamon EOs inhibited the growth of *A. alternata* on solid media.

Nielsen and Rios (2000) reported that oregano EOs exhibit an inhibitory activity against microorganisms commonly associated with bread spoilage. Tepe *et al.* (2004) examined the AM activity of *Thymus eigii* EOs and its main constituents carvacrol, thymol and *p*-cymene against *B. catarrhalis, C. perfringens, B. cereus, Staph. aureus, S. pneumoniae, M. smegmatis* and *P. aeruginosa in vitro* and found that these EOs demonstrate an AM activity against the tested microorganisms. More recently, Gutierrez *et al.* (2008) evaluated the synergistic effect of the EOs of thyme, oregano, lemon balm, marjoram, rosemary and sage against *B. cereus, E. coli, L. monocytogenes* and *P. aeruginosa* using the spot test on agar media. They reported a significant AM activity of oregano in combination with basil, thyme or marjoram against *B. cereus, E. coli* and *P. aeruginosa*. Similarly, Gutierrez *et al.* (2009a) determined the AM activity of the EOs of thyme, oregano, lemon balm and marjoram against *Enterobacter* sp., *Listeria* sp., *Lactobacillus* sp. and *Pseudomonas* sp. using foods based on lettuce, meat and milk. Their findings demonstrated that minimum inhibitory concentrations were significantly lower in lettuce and beef media than in tryptic soy broth and that oregano and thyme were the most active EOs. Lopez *et al.* (2007b) reported AM activity of oregano, cinnamon and clove EOs incorporated into PP or PE/EVOH against various gram negative bacteria (*E. coli, Y. enterocolitica, P. aeruginosa* and *Sal. choleraesuis*), gram positive bacteria (*L. monocytogenes, Staph. aureus, B. cereus* and *E. faecalis*), yeasts (*C. albicans, D.*

hansenii, Z. rouxii) and moulds (*B. cinerae, A. flavus, E. repens, p. roqueforti, P. islandicum, P. commune, P. nalgiovensis*). Similarly, Lopez *et al.* (Lopez *et al.*, 2007a) reported AM activity of cinnamon, oregano and thyme EOs against various gram negative bacteria (*E. coli, Y. enterocolitica, P. aeruginosa* and *Sal. choleraesuis*), gram positive bacteria (*L. monocytogenes, Staph. aureus, B. cereus* and *E. faecalis*), yeasts (*C. albicans)* and moulds (*A. flavus, P. islandicum*). The main constituents: cinnamaldehyde, carvacrol and thymol also demonstrated inhibitory effect against *L. monocytogenes, Sal. choleraesuis, A. flavus* and *C. albicans* using a modified vapour diffusion test.

Sagdiç and Özcan (2003) investigated the AM activity of various EOs including oregano and thyme EOs against different microorganisms including *Bacillus amyloliquefaciens, B. cereus, Enterobacter aerogenes, E. coli, Sal. enteritidis, Staph. aureus* and *Yersinia enterocolitica*. They reported that oregano was particularly effective against all bacteria during incubation. Oussalah *et al.* (2006) studied the inhibitory effects of sixty different EOs including oregano and thyme against *Pseudomonas putida*. The results of their study showed that many EOs possess *in vitro* antibacterial activity against *P. putida* with oregano and thyme EOs demonstrating the highest AM activity. Viuda-Martos *et al.* (2008) evaluated the effectiveness of the EOs of oregano, sage, clove, thyme, rosemary and cumin on the growth of various microorganisms including *Lactobacillus curvatus, Lactobacillus sakei, Staph. carnosus* and *Staph. xylosus, Enterobacter gergoviae* and *Enterobacter amnigenus*. They found that each of the EOs demonstrated inhibitory activity against all bacteria tested with oregano showing the highest AM activity. They reported that the effects of thyme, sage

and rosemary were concentration dependent. Baydar *et al.* (2004) studied the antibacterial activity of EOs of thyme, oregano and savoury against various pathogenic bacteria including *B. cereus, E. coli* and *L. monocytogenes.* They reported positive AM activity against the tested bacteria and suggested that the inhibition may be attributed to the action of the components carvacrol, γ-terpinene and *p*-cymene (a constituent of cumin or thyme EOs). The results of these studies demonstrate that oregano and thyme EOs have the potential to be used as AM agents in the food industry for better preservation of quality, enhancement of safety and extension of shelf life. Nevertheless, additional information is required on the benefits of these EOs before considering them as potential candidates for manufacturing of AM films with commercial applications.

Antimicrobial Efficacy of Carvacrol and Thymol

Carvacrol and thymol are the major components of oregano and thyme EOs. They have received substantial attention as useful natural AM agents due to their natural origin and GRAS status, as well as due to exhibiting a broad AM spectrum against different microorganisms, and possessing heat stability when incorporated into packaging materials (Azaz *et al.*, 2005; Couladis *et al.*, 2004; Deans & Ritchie, 1987; Han, 2005a; Lorenzo *et al.*, 2003; Matan *et al.*, 2006; Ultee *et al.*, 1998; Zaika, 1988). Table 2.4 shows that carvacrol and/or thymol can be applied in food products to control microbial contamination by various microorganisms including bacteria, yeasts and moulds.

Bagamboula *et al.* (2004) determined the AM effect of carvacrol or thymol against *Shigella* sp. (*S. sonnei* and *S. flexneri*) on lettuce. They observed a decrease in *Shigella*

sp. after washing the lettuce with 0.5% and 1% (v/v) thymol or carvacrol and found that at 1% (v/v) of each agent, the population decreased to an undetectable level. They also reported significant inhibition of *Shigella* sp. using the agar diffusion method. The AM activity of carvacrol has also been reported by Ultee *et al.* (2000) when studying the preservation of rice against *B. cereus*. Roller and Seedhar (2002) investigated the effectiveness of carvacrol against the natural flora of freshly cut melons and kiwifruit. They found that carvacrol reduced significantly the viable count of natural flora on kiwifruit dipped in a solution of the agent, but it was less effective on honeydew melons. Kiskó and Roller (2005) explored the AM effectiveness of carvacrol against *E. coli* inoculated into unpasteurised apple juice. They found that carvacrol reduced the bacteria to an undetectable level within the first two days of storage. Ultee and Smid (Ultee & Smid, 2001) found carvacrol to be effective against *B. cereus* toxin production in soups to an undetectable level. Chiasson and others (2004) reported effective AM activity of carvacrol and thymol against *E. coli* and *Sal. typhimurium* in minced meat products. Seaberg *et al.* (2003) reported inhibitory effects of carvacrol against *L. monocytogenes* also in ready-to-eat beef slices. Recently, Rardniyom (2008a) coated carvacrol onto LDPE and nylon films and reported that the AM film inhibited the growth of *E. coli* on Cheddar cheese by log 2.3 and 1.8 CFU g^{-1} on samples stored at 8 and 12°C respectively for 15 days.

In addition to studies on real foods, several studies have reported the inhibitory effect of carvacrol and thymol both on solid and liquid media as shown in Table 2. On solid media, using the agar diffusion test, López-Malo *et al.* (2005) found that carvacrol and thymol had a significant inhibitory effect against *A. flavus*. Singh *et al.* (2006)

investigated the AM activity of thymol against various microorganisms using the agar well diffusion method and showed that thymol inhibited completely the growth of *B. cereus* and *P. aeruginosa*. Tepe *et al.* (2004), reported the positive AM activity of carvacrol and thymol against *B. catarrhalis, C. perfringens, B. cereus, Staph. aureus, S. pneumoniae, M. smegmatis* and *P. aeruginosa in vitro*. Sivropoulou *et al.* (1996) reported significant AM activity of carvacrol and thymol against *S. aureus*. Dorman and Deans (2000) reported effective AM activity of thymol and carvacrol against selected microorganisms including *B. cereus, S. aureus, L. monocytogenes, E. coli, A. niger* and *S. cerevisiae* using the agar well diffusion method. Olasupo *et al.* (2003) reported that carvacrol and thymol demonstrated the highest AM activity against *E. coli* and *Sal. typhimurium* using liquid culture compared to other agents including eugenol, nisin, cinnamic acid and diacetyl compounds. Rupika *et al.* (2005) found that carvacrol and/or thymol impregnated into LDPE films had a significant inhibitory activity against *E. coli, Staph. aureus, L. innocua, P. aeruginosa, A. niger* and *S. cerevisiae* using the agar disc diffusion assay. Han *et al.* (2005c) investigated the effectiveness of carvacrol and thymol coated onto LDPE film against *L. innocua* and *E. coli* in solid and liquid media and observed an inhibitory effect using the agar diffusion method. In the liquid culture test, carvacrol and thymol incorporated into the film reduced significantly the specific growth rate and the final cell concentration of *L. innocua*.

Falcone *et al.* (2005) reported that thymol inhibited significantly the growth of *S. cerevisiae* and *B. cereus* in liquid media. They reported that the growth kinetics of *B. cereus* in liquid media is a function of thymol concentration. Ultee *et al.* (1998) investigated the AM activity of carvacrol against *B. cereus* using a liquid culture media

and reported that the activity depends on the concentration, exposure time, temperature and pH. Periago *et al.* (2004) studied the AM activity of carvacrol and cymene against the growth of two strains of *L. monocytogenes* and found that carvacrol and cymene reduced microbial growth during the lag and exponential phases. They found that the combination of carvacrol and cymene resulted in a larger decrease in viable counts of *L. monocytogenes* compared with the separate application of these agents. Burt *et al.* (2005) conducted a comparative study of the AM activity of oregano and thyme EO components (carvacrol, thymol, *p*-cymene and γ-terpinene) against the growth of *E. coli* O157:H7 and also found synergistic effects of these components using the checkerboard assay. They reported that carvacrol and thymol demonstrated individual and additive antibacterial activity against *E. coli* O157:H7, but no observable AM activity by *p*-cymene and γ-terpinene was found. Although the vast majority of studies involving essential oils or their extracts suggest a positive and broad spectrum of AM activity, an important aspect that needs more attention is how to minimise the loss of these volatile agents during processing, particularly at high temperatures. Gutiérrez *et al.* (2009b) reported AM activity of carvacrol, thymol and cinnamadehyde incorporated into PP against various gram negative (*E. coli, Yersinia enterocolitica, P. aeruginosa* and *Sal. choleraesuis*), gram positive bacteria (*L. monocytogenes, Staph. aureus, B. cereus* and *Enterococcus faecalis*), yeasts (*Candida Albicans, Debaryomyces hansenii, Zygosaccharomyces rouxii)* and moulds (*Botrytis cinerae, A. flavus, Eurotium, repens, penicllium roqueforti, P. islandicum, P. commune, P. nalgiovensis*). Recently, Persico *et al.* (2009) claimed that carvacrol incorporated into a LDPE film demonstrated an AM activity against *B. thermosphacta, L. innocua* and *Carnobacterium. sp* on agar medium.

Other Natural Antimicrobial Agents

Numerous studies have evaluated the inhibitory effects of other natural AM agents including bacteriocins, plant extracts such as grapefruit seed extract (GFSE), enzymes and spices (see Table 2.4). Bacteriocins such as nisin are ribosomally synthesised peptides produced by lactic acid bacteria and possess bactericidal properties against a range of microorganisms (Siragusa *et al.*, 1999). They were widely studied for their AM activity in packaging films. Grower *et al.* (2004) developed an AM film by coating nisin onto an LDPE film and reported that these coatings were effective against *L. monocytogenes* on solid microbiological media and on the surface of individually packed hotdogs. Natrajan and Sheldon (2000b) reported significant AM activity of nisin coated on three different packaging films: polyvinyl chloride (PVC), linear low-density polyethylene (LLDPE) and nylon against *Sal. typhimurium* on broiler drumstick skin stored at 4°C. The AM activity of the nisin film was found to be at higher nisin concentrations and when the film was in direct contact with the tested products for a longer period. Kim *et al.* (2002c) coated nisin onto LDPE film in order to control naturally-occurring bacteria on packaged fresh oysters and ground beef stored at 3 and 10°C. They claimed that nisin coated onto the film reduced microbial growth at both temperatures in contrast to a non-coated LDPE film. The inhibitory effects of AM-coated films on the growth of coliform bacteria were more evident at 10°C than at 3°C, while the effect on the total aerobic bacteria count was consistently apparent at both temperatures. Siragusa *et al.* (1999) evaluated the AM effectiveness of nisin incorporated into LDPE films against the growth of *B. thermosphacta* inoculated on the surface of a beef carcass. They reported that the films reduced significantly the population of *B. thermosphacta* at the end of a storage period at 4 and 12°C. Scannell *et*

al. (2000) investigated the AM activity of nisin immobilised onto polyethylene (PE) and/or nylon films and found that the films reduced the levels of *L. innocua* and *Staph. aureus* in sliced cheese and ham. Mauriello *et al.* (2004) investigated the anti-listerial effect of bacteriocin produced by *Lactobacillus curvatus* 32Y incorporated PE and oriented nylon films. The films were coated with the bacteriocin using three different methods: soaking, spraying and coating and all the films inhibited the growth of *L. monocytogenes* on both solid media and pork steaks. Mauriello *et al.* (2005) coated nisin onto LDPE films in order to control the growth of *Micrococcus luteus* in tryptone soya broth and in raw, pasteurised and UHT milk. The nisin coated onto LDPE films was shown to have an inhibitory effect against the growth of the bacteria in the broth and also reduced microbial counts in the milk products. Cooksey (2001) coated nisin onto LDPE films and evaluated their inhibitory effect against *L. monocytogenes* on packaged hotdogs and reported that coatings containing 2500 IU mL^{-1} or greater of nisin applied to the films effectively inhibited microbial growth on the hotdogs stored under refrigeration for 60 days. Cutter *et al.* (2001) investigated the AM activity of nisin incorporated into PE food packaging films and reported a significant AM effect of the films against *B. thermosphacta*.

The AM activity of plant extracts was investigated by several researchers and invariably demonstrated an inhibitory effect against various microorganisms. For example, Hong and *et al.* (2000) investigated the AM effectiveness of 5% (w/w) propolis extract or clove extract incorporated into LDPE films against *E. coli*, *L. plantarum*, *S. cerevisiae* and *Fusarium oxysporum*. All extracts demonstrated an inhibitory activity on the growth of *L. plantarum* and *F. oxysporum*. Ha *et al.* (2001) investigated the AM activity of

GFSE incorporated into multi-layered PE films against *M. flavus, E. coli, Staph. aureus* and *B. subtilis* on ground beef. The coated films demonstrated an AM activity against all the microorganisms studied. Lee *et al.* (1998) developed an LDPE packaging film incorporated with GFSE and reported that the film containing narigin, ascorbic acid, hesperidin and various organic acids was shown to possess a wide spectrum of AM activity. However, although the LDPE films containing GFSE had an inhibitory effect on the growth of *E. coli* and *Staph. aureus* on solid media, they were unable to inhibit the growth of *Leuconostoc mesenteroides, S. cerevisiae, Aspergillus oryzaei, A. niger* and *Penicillium chrysogenum.* Rodriguez *et al.* (2008) investigated the AM activity of an active packaging film incorporated with cinnamon EOs against *Rhizopus stolonifer* both *in vitro* and on sliced bread. They reported that cinnamon EOs inhibited the growth of *R. stolonifer* on solid media and on sliced bread.

Although AM agents such as EOs and/or their principal components may exhibit AM activity against various microorganisms when incorporated into packaging materials, the organoleptic properties of the packaged food products is one of the important factors that must also be taken into consideration. According to Davison and Zivanovic (2003), the concentration of AM agents required to demonstrate AM activity against various microorganisms on food products might be higher than the concentration applied for flavouring purposes. As a result, this might cause food tainting and/or adverse sensorial effects to food products (Bagamboula *et al.*, 2004; Smith-Palmer *et al.*, 2001). The adverse sensorial effects of AM agents to food products can be overcome by masking the odour of AM agents with other approved aroma compound as suggested by Gutiérrez *et al.* (2009b). The understanding of the relationship between minimum

inhibitory concentration (MIC) and an acceptable organoleptic properties of AM Agents such as EOs and/or their constituents is important (Lambert *et al.*, 2001). In some cases, the replacement of EOs with one or a number of their principal constituents may provide equal AM effectiveness but with milder flavouring attributes (Lambert *et al.*, 2001; Smith-Palmer *et al.*, 2001).

2.12 Potential Applications of AM Films For Cheese Packaging

Different cheeses are products that are highly susceptible to microbial and fungal deterioration facilitated by high moisture content. Microbial deterioration of a cheese product may cause negative health effects and/or pose a health risk to consumers exposed to such contaminated products (Natrajan & Sheldon, 2000b; Padgett *et al.*, 1998). Cheeses are classified into soft and hard cheeses based on how the curd is formed and this classification depends on the texture and on the microbes responsible for ripening the product (Robertson, 1993). Soft cheeses such as cream cheese is characterised by its high moisture content *ca*. 55-80% w/w (Fox & McSweeney, 2004; Kosikowski & Mistry, 1997) and hard cheeses such as Cheddar cheese are characterised by a moisture content of 26-50% (w/w). Many hard cheeses like Cheddar cheeses are packaged in pouches and vacuum sealed which provides protective functions only (Rupika *et al.*, 2005; Suppakul, 2004). Although hard cheeses such as Cheddar cheese blocks have low moisture content, they may also be implicated in microbial contamination and disease outbreaks as in the case of soft cheeses if there is loss of vacuum or improper handling. Given that Cheddar cheese can be implicated in microbial spoilage and/or contamination, its storage may benefit from AM packaging using natural AM agents. Pathogenic and spoilage bacteria such as *E. coli* O157:H7, *S.*

aureus, S. typhimurium, L. monocytogenes have a negative impact on cheese quality and are a risk to human health posing the greatest risk to the safety of dairy products (Fox & McSweeney, 2004; Rodriguez *et al.*, 2005; Virto *et al.*, 2006). Microbial spoilage of Cheddar cheese can also be caused by yeasts (*Candida, S. cerevisiae*) and moulds (*Penicillium, Aspergillus* and *Cladosporium cladosporioides, Penicillium commune, C. herbarum, P. glabrum* and a *Phoma* species) (Hayes, 1992). Moulds can produce discolouration on the surface of cheese blocks (Blank *et al.*, 1992; Hockinga & Faedo, 1992). Various studies have identified various forms of cheeses as the potential food products that can benefit from AM packaging applications (Han, 2005a; Kuorwel *et al.*, 2011b; Rardniyom, 2008a; Rupika *et al.*, 2005; Suppakul *et al.*, 2008; Weng & Hotchkiss, 1992).

Furthermore, many other studies have demonstrated that the basil, oregano, thyme EOs along with their principal constituents linalool, carvacrol and thymol have a potential for food preservation. The AM films incorporated with EOs and/or their principal constituents have also the potential for packaging of many food products such as bakery (Mehyar *et al.*, 2011; Rodríguez *et al.*, 2008; Rokchoy *et al.*, 2009; Suhr & Nielsen, 2003), meat, chicken and fish products (Kerry *et al.*, 2006; Shen *et al.*, 2010; Suppakul *et al.*, 2003a; Wu *et al.*, 2010), and fresh produce (Rodriguez-Lafuente *et al.*, 2010). There are already some commercial applications for AM packaging systems such as wasabi extract (or Japanese horseradish) manufactured by MicroGard™ and Piatech-by Rhone-Poulene (USA) and Daikoku Kasei Co (Japan) and used for Japanese rice lunch boxes (Brody *et al.*, 2001).

3 Materials and Methods

3.1 Materials

3.1.1 Polymers

A commercial starch-based film (Biograde B-F) supplied by Biograde Ltd., Australia was used in this study. Biograde B-F is a biodegradable material based on a blend of thermoplastic starch, aliphatic polyesters and natural plasticisers. The resin is a corn starch based material and in this sense it is a renewable material. Biograde is not considered to be a fully biodegradable material. Another material used in the present study was an hydroxypropylated thermoplastic (TPS) corn starch (Gelose 939) material that has an amylose content of *ca*. 80% (Chaudhary *et al.*, 2009). The starch-based material was supplied by Penford Australia, Ltd. (Sydney, Australia) and has been specifically designed for the production of extruded or thermoformed packaging products. The moisture content of GELOSE 939 determined by weight loss on drying at 70°C under vacuum for 3 days, was 13.3% before processing (Chaléat *et al.*, 2012). The polymers used in the coating solutions were methylcellulose (MC, 18,804-2); hydroxypropyl methylcellulose (HPMC, 42,321-1) and polyethylene glycol (PEG, 20,236-3) were purchased from Aldrich Chemical Company Inc., Milwaukee, WI., USA. The properties of the MC and HPMC are presented in Appendix A.1 and A.2, respectively.

3.1.2 Antimicrobial Agents

The AM additives were linalool (L2602) with a purity of 97%; carvacrol (W224502) with a purity of 98% and thymol (TO501) with a purity of 99.5%. They were obtained from Sigma-Aldrich Pty. Ltd., Australia. The properties of these AM agents (linalool, carvacrol and thymol) are presented in Appendix A.3, A.4 and A.5 respectively.

3.1.3 Media and Count Plates

The media used were nutrient agar (AM 130), potato dextrose agar (AM 149), plate count agar (AM 144), malt extract agar (AM 109) and malt extract broth (AM 110) purchased from Amyl Ltd., Australia. Bacteriological peptone (LP0037) was purchased from Oxoid Ltd., Hampshire, England. The 3M Petrifilm™ yeast and mould count plate (6417) and 3M Perifilm™ Staph Express Count Plate (6490) purchased from 3M Microbiology Products, USA. For the preparation of diluents in microbial assay sodium chloride (567440) and sodium hydroxide (NaOH) (410203) were purchased from Merck™ Chemicals, Australia.

3.1.4 Microorganisms

The microorganism *Staphylococcus aureus* (UNSW 056201), *Saccharomyces cerevisiae* (UNSW 703100) and *Aspergillus niger* (UNSW 80900) were obtained from the culture collection of the University of New South Wales, Australia. The detail characteristics of the tested microorganisms are presented in Appendix A.6.

3.1.5 Other Materials

The solvents used include ethanol (absolute, AR grade) supplied by Merck Pty. Ltd., Australia, and isooctane (2, 2, 4–trimethylpentane, OmniSolv®, TX 1389-1) supplied by EMD™ Chemicals Inc., USA, were used as the solvents. Commercially produced matured Cheddar cheese (matured 6-9 months) was purchased in blocks (10 × 1 kg) from a commercial retail outlet. The blocks were packaged in pouches, vacuum-sealed and all had the same date code from the same batch. According to the manufacturer, a 100 g sample of cheese contains the following main components: fat 35.2 g; protein 24.3 g; carbohydrates 0.1 g; calcium 735 mg and sodium 635 mg. The blocks were cut to the appropriate size pieces for experimentation.

3.2 Methods

3.2.1 Preparation of TPS Film

The preparation of starch-based films was achieved by heat pressing in accordance with the method previously used by Mistry (2006). Masterbatches were prepared by gradually adding the starch-based material to a plasticiser made of a mixture of water and glycerol. The final composition of the formulations was 65% (w/w) starch-based material, 10% (w/w) water and 25% (w/w) glycerol. A sample weighing *ca.* 15 g of the resultant mixture was then placed between Mylar™ films that were then positioned between a set of aluminium plates and pressed in a laboratory press (IDM Instruments Pty. Ltd., Australia, Model No. L0003). The temperature of the upper and lower platens of the press was maintained at 125°C for 5 min under a pressure of 20 kPa. The plates

were quench-cooled, removed from the press and the films were peeled off the Mylar™ substrate after cooling was completed. The TPS Films thicknesses were measured using a hand-held micrometer with a precision of 0.001 mm (Mitutoyo, Japan). The film thickness was measured at 5 different positions and the average thickness was calculated from these readings.

3.3 Characterisation of Starch-Based Films

3.3.1 Water Sorption Measurements

The water uptake of TPS and APTPS films was measured by: (i) directly immersing film pieces into isopropanol-water, ethanol-water and glycerol-water mixtures and (ii) conditioning film samples at different levels of RH. In the immersion tests, film pieces were directly immersed into mixtures of 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and 0% (v/v) water in isopropanol, water in ethanol and water in glycerol for 5 min at temperature of 20 ± 1°C. Samples were then removed and wiped dry and the final weight was determined using an analytical balance. To test the effect of RH, film samples were contained in desiccators and exposed for 7 days over saturated solutions of P_2O_5, LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, $NaNO_2$, NaCl, KCl, and K_2SO_4 in distilled water to achieve relative humidities of: 0, 11, 23, 33, 43, 53, 66, 75, 85, 97 and 100% respectively at the temperature of 20 ± 1°C (Mathlouthi, 2001). The water content of the equilibrated film samples was determined gravimetrically by firstly weighing the exposed samples and then drying them at 105°C in a laboratory oven for 24 h before re-weighing.

3.3.2 Mechanical Properties of Starch-Based Films

The effect of water content and RH on the mechanical properties of TPS and APTPS starch-based films was investigated by measuring the tensile properties in accordance with ASTM Method D 882-97. Films were cut into strips of 20 × 100 mm in dimensions and divided into two sets for the effect of water content and the RH experiments. One set of film strips was directly immersed into mixtures of isopropanol/water, ethanol/water and glycerol/water for 5 min. Another set of film strips was conditioned for 7 days at 20 ± 1°C over the various saturated salt solutions used for the water uptake experiments. The tensile strength of the film section was determined using an Instron 4465 (USA) tensile tester with an R 2797 (500 N) peak load cell and speed of 50 mm min^{-1} and 500 mm min^{-1} for the TPS and APTPS starch-based films respectively. Five replicates were tested for the tensile measurements to establish average values for tensile strength, elongation at break and the modulus of elasticity.

3.4 Data Analysis

Data points represent the mean value of the results obtained for the TPS and APTPS films. Five replicates were taken in the case of the determination of the mechanical properties of the films. A total of three replicates were used for the water sorption determination in each of the immersion and RH exposure tests. Data points were subjected to analysis of variance (ANOVA) using the general linear model procedure of the SAS statistical package (SAS version 9.5, SAS Institute, Cary, NC). Differences amongst the results were examined by the least significant differences test at a probability level of $p = 0.05$.

3.4.1 Preparation of Antimicrobial TPS Film

The TPS films were incorporated with the natural AM agents: linalool, carvacrol and thymol at three different concentrations. The preparation of AM TPS films was achieved by heat pressing. The final composition of the formulations was 63%, 61% or 59% (w/w) starch-based material, 10% (w/w) water, 25% (w/w) glycerol and the remainder AM agent. Each of the three natural AM agents, carvacrol, thymol and linalool was incorporated into the starch-based material individually at one of three different formulation concentrations of 2%, 4% or 6% (w/w). Films samples were heat pressed in accordance with the conditions method in section 3.2.1. A control film without AM agents was produced in a similar manner. Film thickness of the AM TPS films were measured using a hand-held micrometer as described in Section 3.2.1 After measuring the thickness, the films were wrapped in aluminium foil to prevent further loss of the AM agent before being used.

3.5 Quantification of AM agents in the TPS Films

The compression moulded starch-based film samples of approximately 5 × 5 cm were immersed in a sealed vessel of 100 mL of isooctane and were placed in an incubator shaker (Innova^{TM} 4230, New Brunswick Scientific, U.S.A.) and maintained at 37°C. The concentration of AM agent that had migrated from the starch-based film into 100 mL of isooctane as a function of time was analysed by gas chromatography (GC). The quantification of AM agents in the heat pressed and coated film were determined following the method used by Suppakul (2004) and Rupika (2008) where the robustness of the procedure and validation of the method has been previously determined. In using

the method steps were taken to ensure that all data were collected from within the linear calibration range. As the measurements were well within the calibration range it was deemed unnecessary to explore the detection limits of the method.

3.5.1 Gas Chromatographic Analysis

A gas chromatograph (Varian Star 3400-CX GC) system equipped with a fused silica capillary column (DB-5: 30 m × 0.25 mm i.d., film thickness 0.25 μm, J & W Scientific, USA) was used. The conditions applied in the GC were as follows: injected volume: 1.0 μL; initial column temperature: 80°C; heating rate: 5°C min^{-1} up to 120°C, held at this temperature for an additional 10 min; injector temperature: 250°C; FID detector temperature: 300°C; flow rate: 15 mL min^{-1}; splitless; carrier gas: nitrogen. The AM agent contents of samples were calculated from prepared standard curves.

3.5.2 Scanning Electron Microscopy of Starch-based Films

Scanning electron microscopy (SEM) was used to determine the morphology of the film samples. Images of the control APTPS film, control TPS film and TPS film incorporated with linalool, carvacrol and thymol at a formulation concentration of 2, 4 or 6% (w/w) were obtained using a JOEL NeoScope (JCM-5000) scanning electron microscope. Samples were mounted on an aluminium sample holder and coated with up to 6 nm of gold using a NeoCoater (MP19020NCTR) coater prior to acquiring the SEM images at 15000 x magnification under high vacuum and using an accelerating voltage of 10 kV.

3.5.3 Effects of AM Agents on Mechanical Properties of TPS Films

The effect of AM agents (linalool, carvacrol and thymol) on the mechanical properties of TPS films was investigated by measuring the tensile properties in accordance with ASTM Method D 882-97. Films were cut into strips of 20 × 100 mm in dimensions. The tensile strength of the AM TPS film was determined using an Instron 4465 (USA) as described in Section 3.3.2. The tensile strength of the TPS film section was determined using a crosshead speed of 50 mm min^{-1}.

3.5.4 Effects of AM agents on Water Vapour Permeability

The water vapour permeability (WVP) of the TPS films containing AM agents was determined using the modified ASTM E96-93 method (McHugh & Krochta, 1994). Following the method used by Olivas & Barbosa-Cánovas (2008), film samples were sealed to cups containing anhydrous calcium sulfate (0% RH). The initial weight of the cups was determined before being placed in an air-circulating oven at 25°C. The RH of the oven was kept at 75% using a saturated solution of sodium chloride (Alves *et al.*, 2007) throughout the time the samples were exposed. The weight gain of the cup was determined daily for 5 days using an analytical balance. The water vapour transmission rate (WVTR), in units of g day^{-1} m^{-2}, for each film treatment was calculated using equation (3.1):

$$WVTR = W/(t \times A) \quad (3.1)$$

where W is the mass gained, t is the time and A is the area of the exposed film. The ratio W/t was obtained from a linear regression analysis of the weight gain versus time data over a constant period.

The water vapour permeability (WVP) in units of g day^{-1} m^{-1} kPa^{-1} was calculated using equation (3.2):

$$WVP = WVTR \times l/\Delta P \quad (3.2)$$

where, l is the film thickness and ΔP is the water vapour pressure differential across the film (Du *et al.*, 2008a; Mali *et al.*, 2004). The water vapour pressure differential across the film was determined from water vapour pressure of anhydrous calcium sulfate (0% RH) and sodium chloride (75% RH). The WVP values were calculated following the WVP Correction Method used by Gennadios *et al.*(1994).

3.5.5 Optical Properties of Antimicrobial TPS Films

The transparency of the TPS films incorporated with AM agents was determined by using a UV spectrophotometer (UV-1800, Shimadzu Corp, Kyoto, Japan). A rectangular piece of film was cut and directly placed into the spectrophotometer cell with air as a reference (Rao *et al.*, 2010). The transmittance of the each sample was scanned from 190 to 800 nm (Fang *et al.*, 2002; Zhang & Han, 2006). The transparency was calculated by measuring the percentage transmittance of light at a wavelength of 600 nm (Han & Floros, 1997).

3.5.6 Thermal Properties of Antimicrobial TPS Films

The thermal properties of the starch-based films were investigated using a Perkin-Elmer DSC-7 differential scanning calorimeter (DSC) in accordance with ASTM Method D3417-83. Film samples of *ca.* 10-15 mg were weighed into an aluminium pan. In accordance with Suppakul *et al* (2006), nitrogen was used as the purging gas for all the DSC measurements. An empty aluminium pan was used as a reference. The crystalline melting temperature (T_m) was determined by heating the samples from 30°C to *ca.* 250°C at a rate of 10°C min^{-1} and with a flow rate of nitrogen of 20 mL min^{-1}. The T_m of each sample was determined from the temperature axis on its thermogram and T_m was taken at the maximum of the endothermic peak.

3.6 Data Analysis

Data points represent the mean value of the results obtained for the AM TPS films. Five replicates were taken in the case of the effects on the mechanical properties of the films. Experiments on WVP, thermal properties and light transmission were performed in duplicate for each formulation of film. Data points were subjected to analysis of variance (ANOVA) using the general linear model procedure of the SAS statistical package (SAS version 9.5, SAS Institute, Cary, NC). Differences amongst the results were examined by the least significant differences test at a probability level of $p = 0.05$.

3.7 The Coating of Antimicrobial Films

A coating procedure described by Rardniyom (2008a) was used for coating the starch-based films. The coating solution was prepared by dissolving MC and HPMC in absolute ethanol. The mixture was then heated with magnetic stirring on a hotplate. The heating was discontinued when the temperature reached 65°C. With continuous agitation, a mixture of PEG and distilled water was slowly added to the MC-HPMC dispersion as a plasticiser whilst the dispersion cooled and this resulted in the formation of a uniformly clear coating solution or gel. The AM agent was then added to the coating solution at three different concentrations resulting in the final coated material with concentrations of 1, 3 or 5% (w/w) of AM agent. The coating medium was applied to the starch-based material using a hand-drawn glass roller and the film was then dried under ambient conditions (21°C) for 24 h. To control the thickness of the coated films, the starch-based medium was taped onto a 30 × 30 cm glass plate with a perimeter border created around each edge using 3M™ masking tape. Similarly, a starch-based film whose coating contained no AM agent was prepared as the control.

The addition of AM agent and subsequent coating procedures were conducted at ambient temperature to minimise the loss of the volatile additives from the coatings. The total film thickness of the coating and starch-based film was measured immediately after peeling them off using a hand-held micrometer with a precision of 0.001 mm (Mitutoyo, Japan). The film thickness was measured at 5 different positions and the average thickness was calculated from these readings. After measuring the thickness, the films were wrapped in aluminium foil to prevent further loss of the AM agent before being used. Although ethanol is an AM agent, its presence was assumed to be at very

low level due to evaporation during the 24 h period. Therefore, its presence would have no major effect on the AM activity of the films.

If one sprays the surface of packaging material with solutions of these AM agents, there would be a possibility of short term protection due to volatility of AM agents used in this study. The coated material would retained significant amount of volatile AM agents compared to when these AM agents are incorporated into packaging materials using extrusion and compression moulding techniques that utilised harsh processing conditions such as high processing temperature and/or shear forces. Coated packaging material can provide long term protection of a packaged foodstuff because the AM agents coated onto the packaging material would be released slowly onto the surface of the packaged foodstuff.

3.8 Quantification of AM agents in the MC-HPMC Coatings

The glass plates were coated with MC-HPMC coating medium containing AM agents (linalool, carvacrol and thymol) prepared in accordance with the method described in Section 3.2.2. The dried coated glass plates were immersed in 100 mL of 95% (v/v) ethanol in a closed container at ambient temperature for 24 h and were shaken on an automatic shaker with a rotating speed of 60 rpm. The concentration of AM agent that had migrated from the dried MC-HPMC coatings into the 95% (v/v) ethanol was analysed by GC as described in Section 3.5.1. The concentration of the AM agents was also determined in terms of the total film weight (MC-HPMC coatings + starch-based film) in grams. The concentration of each AM agent was measured in units of g/cm^{-3}.

The actual percentage of AM agents in the coated APTPS film was then determined on the basis of total dry weight of coating materials (MC-HPMC coatings) and APTPS film.

3.9 The Migration of Antimicrobial Agents from Starch-Based Films

3.9.1 Migration of Antimicrobial Agents into Food Simulant

The release of AM agents (linalool carvacrol or thymol) from heat pressed starch-based film samples into a food simulant were studied at three temperatures 15, 25 and 35°C. The migration was examined using a total immersion migration test with isooctane as a fatty-food simulant (EC, 1997; FDA, 2007). Film samples weighing *ca*. 0.5 g were immersed in 100 mL of isooctane in a tightly sealed vessel that was gently agitated (60 rpm) in an incubator shaker (InnovaTM 4230, New Brunswick Scientific, USA). The release of AM agents from the MC-HPMC coated APTPS films was also investigated at each of the temperatures used in the heat pressed samples. The amount of AM agent released from the pressed starch-based films and/or MC-HPMC coatings were monitored until equilibrium was attained. The amount of AM agent released from these systems at particular time intervals was analysed by GC in accordance with the conditions described in Section 3.5.1.

3.9.2 Migration of Antimicrobial Agents to Atmosphere

The retention of AM agent in the MC-HPMC coated APTPS films was investigated under two different storage conditions: (i) exposure to air at room temperature and (ii)

wrapped in foil and stored at room temperature during 28 days of storage. Aluminium foil is considered to be an absolute barrier to aroma (Lamberti & Escher, 2007). The AM films were analysed for the retained AM agent at regular intervals using a Fourier transform infrared (FTIR) spectrophotometer (Bruker model Vector 22). Fourier transform infrared spectroscopy is one of the well established analytical techniques that can be used for quantifying the amount of AM agents that is released to the atmosphere provided that a spectrally distinct absorption that is associated with the analyte exists. The FTIR technique can be used to identify the functional groups associated with the structures of essential oil components such as carvacrol and thymol (Schulz *et al.*, 2003). Moreover, the technique can be used as a qualitative and quantitative tool in many applications including the study of the transport of aromatic compounds through polymeric films (Cava *et al.*, 2005; 2004) and the determination of the concentration of additives in LDPE (Graciano-Verdugo *et al.*, 2006). The existing literature also demonstrates that the relative loss of linalool, carvacrol and thymol to the atmosphere during storage can be determined by monitoring an appropriate infrared (IR) peak due to the AM agent and measuring the change in absorbance of the peak over time (Rardniyom, 2008a; Rupika, 2008a). For example, carvacrol has an IR absorbance band that has a maximum at wave number 3450 cm^{-1}, corresponding to its hydroxyl group. The peak height of the absorbance measured at this wavenumber is proportional to the mass fraction of the AM agent retained in the films. Consequently, the FTIR technique was used in this study to quantify the stability of AM films because it is a rapid and non-destructive analytical technique. Other analytical techniques such UV-Vis, NIR, GCFID or GCM can also be used for quantifying the AM agents during long-term storage.

The IR transmittance spectra were measured in the wavenumber range of 4000 to 370 cm^{-1} (32 scans at a resolution of 4 cm^{-1}). The wavenumber of the spectra that corresponded to carvacrol absorption was selected and its peak height was recorded. The concentration of the AM agent that was retained in the films was calculated as a proportion of the initial concentration. The post-processing concentration of carvacrol in the MC-HPMC coatings, determined by GC was taken as the initial concentration in the films prior to storage on day 0. The standardised data for each day of analysis was converted to absolute values by division of the ratio for each analysis day by the ratio on day 0. The results were then multiplied by the initial carvacrol concentration to obtain residual concentration (% w/w) of carvacrol in the films on each day of analysis. Results were expressed as percentage of AM agent remaining in the film and were plotted as a function of time (d).

3.10 Data Analysis

The migration of AM agents (carvacrol, thymol or linalool) from the starch-based film was analysed using two data analysis treatments, namely the overall kinetics and the diffusion models in accordance with Cran *et al*. (2010). Equations describing the migration of AM agents from a polymeric film with time have been described by Miltz (1987) and Crank (1975).

The release of AM agent into the food simulant was initially analysed for the fit to first-order kinetics model. For a first-order system, equation 4 is used:

$$ln(1-\frac{m_t}{m_\infty}) = -k_1 t \qquad (3.3)$$

where k_1 is the first-order rate constant. From equation 3.3, a plot of $\ln(1 - m_t/m_\infty)$ (as previous comment) versus time over the entire time domain should follow a straight line with a slope, of $-k_1$.

The initial rate of release of the AM agent, v_0, at time $t = 0$, is therefore given by:

$$v_0 = m_\infty k_2 \tag{3.4}$$

For the kinetic approach to data analysis, the rate constants were calculated using equation 3.3 and the initial release rates of AM agent were calculated using equation 3.4.

In the diffusion model, the release of the AM agent from the film into a food simulant is considered in two stages, namely the short-term and the long-term. For short-term migration $m_t/m_\infty < 0.6$:

$$\frac{m_t}{m_\infty} = 1 - \frac{8}{\pi^2}\exp\left[\frac{-\pi^2 Dt}{l^2}\right] \tag{3.5}$$

where m_t is the amount of AM agent released from the film, m_∞ is the equilibrium amount of AM agent released from the film at time t, D is the diffusion coefficient and l is the film thickness. A plot of m_t/m_∞ versus $t^{1/2}$ should yield a straight line from which the diffusion coefficient can be obtained. For long-term migration $m_t/m_\infty > 0.6$, equation 3.5 is used:

$$\frac{m_t}{m_\infty} = 1 - \frac{8}{\pi^2} \exp\left[\frac{-\pi^2 Dt}{l^2}\right] \tag{3.6}$$

Rearranging equation 3.6 yields:

$$ln\left[1 - \frac{m_t}{m_\infty}\right] = ln\left[\frac{8}{\pi^2}\right] - k_2 t \tag{3.7}$$

Rearranging equation 3.7 yields:

$$\ln(1 - \frac{m_t}{m_\infty}) = -k_2 t \tag{3.8}$$

where k_2 is the rate constant. From equation 3.8, a plot of $\ln(1 - m_t/m_\infty)$ versus time should yield a straight line with a slope, of $-k_2$. In the case of the analysis according to the diffusion model, the diffusion coefficients were calculated using equation 3.5 for short-term migration and the rate constants were calculated using equation 3.7 for long-term migration.

In order to determine the effect of temperature on the release of AM agents, an Arrhenius activation energy equation was used (Rardniyom, 2008a; Suppakul, 2004). The activation energy of diffusion (E_a) was obtained from D values at different temperatures using equation 3.8:

$$D = D_0 \exp\left(\frac{-E_a}{RT}\right) \tag{3.8}$$

where D_0 is the pre-exponential factor, R is the ideal gas constant, and T is the absolute temperature.

3.11 Antimicrobial Activity of Antimicrobial Films

3.11.1 Preparation of Microbial Inocula

The bacterial and yeast cultures were maintained at -80°C in nutrient broth and malt extract broth containing 30% glycerol. The cultures were sub-cultured in liquid medium twice before being used. A spore suspension of *A. niger* in malt extract broth and 30% glycerol was used for long-term maintenance. For the bacteria and yeast cultures, cell densities of *ca.* 10^6 CFU mL^{-1} were prepared in 0.1% (w/v) sterile peptone solution from early stationary phase cells and confirmed by spread plate count on plate count agar and potato dextrose agar for bacteria and yeast respectively. The fungal species *A. niger* inoculum were prepared by growing spores for 1 week on malt agar at 25°C prior to harvesting and suspension in 5 mL of 0.85% (v/v) saline. The spore density was adjusted to *ca*. 10^6 spores mL^{-1} by serial dilution in 0.85% (v/v) saline. The spore density of the final inoculum was confirmed by colony counts on potato dextrose agar.

3.11.2 Antimicrobial Activity Assay on Solid Media

Agar Disc Diffusion Method

The effectiveness of AM starch-based films on a solid medium was determined using the agar disc diffusion assay in accordance with the method described by Rupika *et al.* (2005) and Suppakul *et al.* (2008). A bacterial (*S. aureus*) or yeast (*S. cerevisiae*) suspension at the level of 10^6 CFU mL^{-1} was prepared in a 0.1% (w/v) sterile peptone solution. The control and AM starch-based films were cut into circular discs (6 mm diameter) and sterilised using UV light for 2 min (Cooksey, 2000). The cut pieces were aseptically placed on nutrient agar or malt agar plates seeded with 0.1 mL of *S. aureus* or *S. cerevisiae* solution. The plates seeded with *S. aureus* were incubated at 37°C for 24 h. The plates seeded with *S. cerevisiae* were incubated at 25°C for 48 h. After the incubation process, the diameters of the clear zones that formed around the film samples were measured using a Vernier calliper and reported as the zone of inhibition. Such a qualitative measurement is sufficient to make meaningful comparisons between the systems studied in the present work. Although not used to analyse the data in the current study, another approach involves the calculation of an antimicrobial index (AM_i) defined in equation (3.9):

$$AM_i = (d_1-d_2)/d_1 \tag{3.9}$$

where d_1 is the diameter of clear zone and d_2 is the diameter of circular film. All the experiments were performed in triplicate and the data are reported as the mean.

Microatmosphere Method

The modified microatmosphere method described by Guynot *et al.* (2003) was used to study the inhibitory efficacy experiments on *A. niger* on solid media. The *A. niger* inoculum was prepared by growing spores from suspensions in glycerol for 1 week on malt agar (Amyl, AM 109) at 25°C. Spores from the new growth were then harvested and suspended in 5 mL of 0.85% (w/v) saline. Malt extract agar plates were prepared and centrally inoculated with 20 μL of the *A. niger* suspension. The inoculated agar plates were kept in the inverted position with the prepared AM films placed in the lid. In each case prior to experimentation, a circular disk (6 mm in diameter) was cut from the coated AM film, sterilised with UV light for 2 min (Cooksey, 2000) and mounted in the centre of the petri dish lid. The lids were then tightly sealed to the plates with Parafilm™ and incubated for 1 week at 25°C. The efficacy was determined by measuring the diameter of the growing fungus colonies on the surface of the agar after 1 week of incubation.

3.12 Antimicrobial Activity on Cheddar Cheese- A Challenge Test

3.12.1 Cheese Preparation

Cheddar cheese was purchased from a local supermarket and cubes of the cheese weighing *ca*. 20 ± 1 g each were cut (Rupika *et al.*, 2005; Suppakul, 2004). Samples were divided into four sets, for the control film and the three AM coated films containing carvacrol, linalool or thymol. The cheese samples were then sterilised on all sides for 1 h using UV light. For sterilisation of the inoculated cheese samples, samples

were irradiated using a germicidal UV lamp. The radiation source was a 30 watts UV germicidal lamp (G30T8, Sankyo Denki, Japan) measuring 88 × 2.5 cm and emitting radiation at a wavelength of 254nm. For power source, the lamp was fixed to the ceiling of Laminar Flow cabinet (Gelman HWS Series, CWS-180, Singapore) used as a test chamber. The control and AM films were also sterilised with UV light prior to use. The exposure of cheese to UV light for 2 h is deemed sufficient to sterilise the cheese samples before inoculation.

3.12.2 Microbial Inoculation on Cheddar Cheese

Each of the cheese samples was inoculated on the top and bottom surfaces with *S. aureus, S. cerevisiae* or *A. niger* and then spread using a sterile glass rod to obtain *ca.* 10^4 CFU g^{-1} (Suppakul *et al.*, 2008) prior to wrapping with the control or an AM test film. The inoculated cheese samples were placed between folded films and then the open sides of the films were sealed. The packaged cheese samples were prepared in duplicate and stored at 15°C for 21, 28 and 35 days for *S. aureus, S. cerevisiae* and *A. niger* respectively. The temperature of 15°C was chosen in order to mimic a temperature abuse condition that might arise in the food supply chain (Siragusa *et al.*, 1999).

3.12.3 Analysis and Enumeration

The analysis of the Cheddar cheese samples was performed periodically on two samples from each treatment that were aseptically opened on the predetermined sampling days. An 11 g sample of cheese was aseptically transferred to a sterile stomacher bag. In

accordance with the method described by Rupika *et al*. (2005), 99 mL of 0.1% (w/v) sterile peptone solvent (pH 7.5 ± 0.2 at 25°C) were added to the sample which was then homogenised using a laboratory blender (Seward Stomach® 400, Seward Medical, UK) for 3 min. Serial dilutions of the resulting solutions were prepared in a sterile peptone diluent (pH 7.0 ± 0.1 at 25ºC) in order to obtain a quantifiable colony count. For the determination of *S. aureus* counts, 1 mL of each serially diluted sample was plated in duplicate on a 3M Perifilm^{TM} Staph Express Count Plate and then incubated aerobically for 24 h at 37°C. To obtain a quantifiable colony count in the case of *S. cerevisiae* and *A. niger*, 1 mL of each serially diluted sample was plated, in duplicate, on a 3M Petrifilm^{TM} yeast and mould count plate and then incubated for 5 days at 25°C. The colonies were counted and the results were expressed as colony-forming units per gram (CFU g^{-1}).

3.12.4 Determination of Microbial Death by AM Films

The death rate of *S. aureus, S. cerevisiae* or *A. niger* inoculated onto the cheese samples during storage was determined in accordance with the calculation procedures described by Bachrouri and others (Bachrouri *et al.*, 2002). Accordingly, a specific death rate, μ, can be determined from equation (10):

$$N = N_0 e^{-\mu t} \qquad (3.10)$$

where, N is the population surviving at any time t, N_0 is the initial population. Taking natural logarithms of both sides of equation (10) results in equation (11):

$$\ln(N) = \ln(N_0) - \mu t \quad (3.11)$$

The specific death rate is obtained from the gradient of a plot of the natural logarithm of N (expressed either in units of CFU mL^{-1} or CFU g^{-1}) versus time. In the present study, the decadic logarithm of the surviving population of *S. aureus, S. cerevisiae* or *A. niger* in the presence of the three different AM agents was plotted as a function of storage time. A linear regression analysis was performed on each curve to obtain the specific death rate, μ', where $\mu' = \mu/\ln(10)$. The values of μ' were subsequently used to compare the AM activity of the films. In the present study, the decadic logarithm of the surviving population of *S. aureus, S. cerevisiae* or *A. niger* in the presence of the three different AM agents was plotted as a function of the time of storage. A linear regression analysis was performed on each curve to obtain the specific death rate, μ. The values of μ were subsequently used to compare the AM activity of films.

3.13 Data Analysis

Experiments on solid media were performed in triplicate for each formulation of film coating. For the analysis of Cheddar cheese, each experiment was replicated twice on different days with two observations per film treatment (n = 4). Individual experiments for each of the AM agents (linalool, carvacrol and thymol) coated onto the starch-based films were performed separately and by comparing the three levels of AM agent added to the starch-based material. Data points are represented by the mean of the results for each AM agent coated onto the substrate. Microbial colony counts were converted into

decadic logarithm values. The latter were subjected to analysis of variance (ANOVA) at the 0.05 confidence level. Differences amongst the treatments were examined by the least significant differences tests using SAS (Version 9.5, SAS Institute, Cary, NC).

4 Results and Discussion

4.1 Characterisation of TPS and APTPS Films

4.1.1 Water Sorption Measurements

The water uptake of thermoplastic starch-based (TPS) and aliphatic thermoplastic starch-based (APTPS) films that were directly immersed in isopropanol/water mixtures is shown in Figure 4.1(a). The TPS film exhibited a significant ($p \leq 0.05$) increase in its absorption of water with increasing water content in the isopropanol/water mixture. The TPS film behaved similarly when immersed in ethanol/water and glycerol/water mixtures (see Appendix B.1).

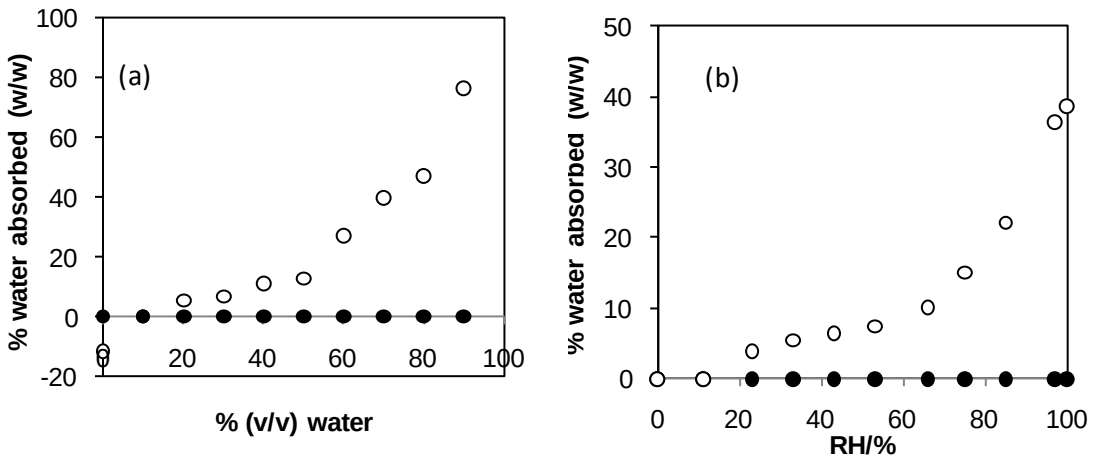

Figure 4.1 Percent water uptake of TPS (○) and APTPS (●) films at 20 ± 1°C as a function of (a) isopropanol concentration in the isopropanol/water immersion mixture after 5 min immersion and (b) RH after conditioning for 7 days.

The plots shown in Figure 4.1(a) indicate that the isotherm obtained for the TPS film is sigmoidal and the water sorption increased steadily with water content in the isopropanol mixture up to *ca*. 40% (v/v) water. When the concentration of water in the mixture was further increased, the TPS material became mechanically unstable and eventually dissolved. The results for the TPS film immersed in ethanol/water mixtures also showed a significant water sorption at elevated water contents in the mixture but in this case there was a steady increase in water content up to *ca*. 20% (v/v) before dissolution of the film became evident.

Interestingly, samples of TPS films immersed in either pure isopropanol or ethanol lost some water, presumably due to water extraction by the isopropanol or ethanol (Lee *et al.*, 1991). Furthermore, it appears that the TPS film does not absorb or lose water at a level of *ca*. 10% (v/v) water in the mixture and this corresponds to the amount of water remaining in the film after heat pressing. The immersion of the TPS films in glycerol/water solutions demonstrated a steady and significant increase in the absorption of water with increasing water content in the mixture up to a level of *ca*. 60% (v/v). When TPS film samples were immersed into pure glycerol, there was no measurable absorption or desorption of water, which is in contrast to the results for isopropanol/water and ethanol/water mixtures. Figure 4.1(a) reveals that the APTPS film behaves in a stark contrast to the TPS film in that the APTPS film did not demonstrate water uptake in any of the isopropanol/water mixtures tested. This behaviour for APTPS films was also observed in the case of the other mixed solvent systems that were tested. The increase of water uptake by the TPS films might be due to microcracks observed at the surface of the films. These microcracks are visible in the

SEM micrograph shown in Figure 4.2(a) and they allow moisture diffusion through the film. In contrast, no microcracks were observed at the surface of APTPS films (see Figure 4.2(b)).

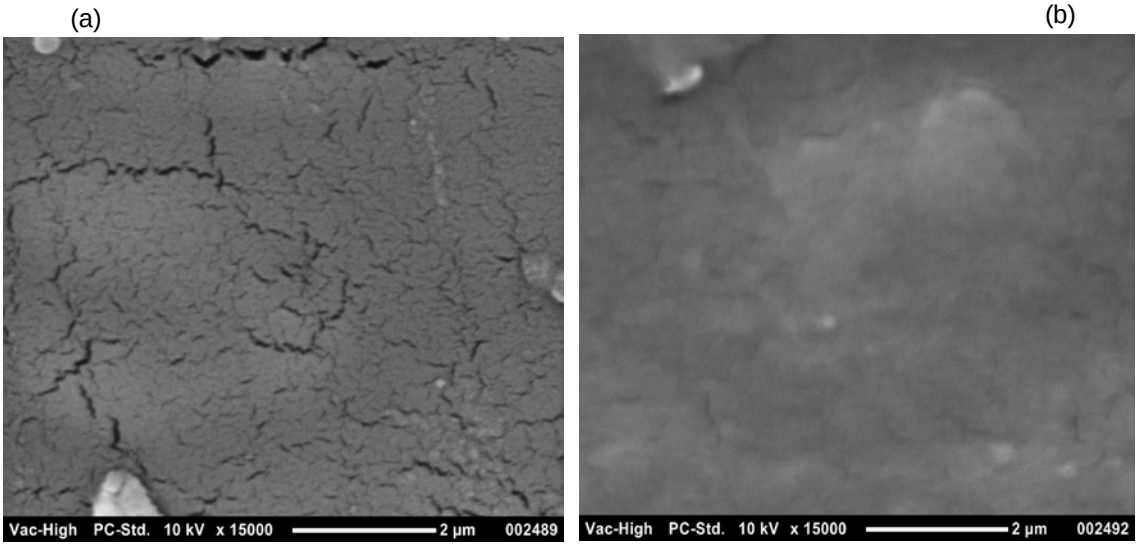

Figure 4.2 The SEM micrographs of: (a) TPS film (b) APTPS film. The SEM images were acquired at 15000 x magnification under high vacuum and using an accelerating voltage of 10 kV.

The water uptake of the TPS and APTPS films conditioned at different RH levels is shown in Figure 4.1(b). The TPS film showed a significant ($p \leq 0.05$) absorption of water with increasing RH. The isotherm obtained for this film exhibits a steady increase in moisture content up to RH ≈ 66% with a rapid rise in the moisture absorption beyond this RH level. This water uptake behaviour is characteristic to TPS materials and results from their hydrophilic nature (Biliaderis *et al.*, 1999; Cho & Rhee, 2004). A similar trend was observed by Van Soest *et al*. (1996) who reported that absorption and

desorption of water by hydrophilic films depend on the RH of the environment. The results of the present study are also in agreement with the findings of Talja *et al.* (2007) who reported higher water sorption with increasing RH by starch-based films. The observed RH dependence of TPS films may be attributable to the high percentage of amylose in the cornstarch (Gelose 939) from which they are comprised. The low moisture uptake of the TPS film at low RH may be due to weak interactions between the water and starch matrix as suggested by Zhang and Han (2008). However, the high moisture uptake exhibited by these films at higher RH is probably due to an increase in hydrogen bonding between starch-water and plasticiser-water, resulting in the weakening of the film structure (Bader & Göritz, 1994; Zhang & Han, 2008). In contrast to the TPS film, no measurable water absorption was observed in the APTPS films upon its immersion or as a function of RH. These results confirm that the hydrophilicity of starch-based packaging materials can be overcome by their chemical modification and/or blending with other polymers (Arvanitoyannis & Biliaderis, 1999; Orts *et al.*, 2007).

4.1.2 Effects of Water Content/RH on Properties of Starch-Based Films

The effect of water content on the ultimate tensile strength (σ), elongation at break (ε_b) and elastic modulus (E') of the TPS and APTPS films was explored by analysing the stress-strain curves of the films. The TPS films when immersed for 5 min in glycerol/water, ethanol/water and isopropanol/water mixtures, all demonstrated a considerable decline in the tensile strength with an increase in the water content in the mixtures as shown in Figures 4.3(a), 4.3(b) and 4.3(c) respectively. In general, the tensile strength of the TPS film is persistent up to *ca*. 50% (v/v), 20% (v/v) and 40%

(v/v) water in the glycerol/water, ethanol/water and isopropanol/water systems respectively. The data obtained for the isopropanol/water systems are comparatively greater than those obtained for the ethanol/water systems presumably as a consequence of the lower polarity of isopropanol compared with ethanol and, to a lesser extent, the relative molecular sizes (Westgate & Ladisch, 1993). Indeed, the presence of water in the solvent/water mixtures enhances the overall polarity of the mixture, resulting in an increased water uptake. Moreover, the larger isopropanol species cannot penetrate the starch film as effectively as ethanol. The ethanol molecules can penetrate the film and extract water molecules present in the starch structure and lead to an onset of brittleness.

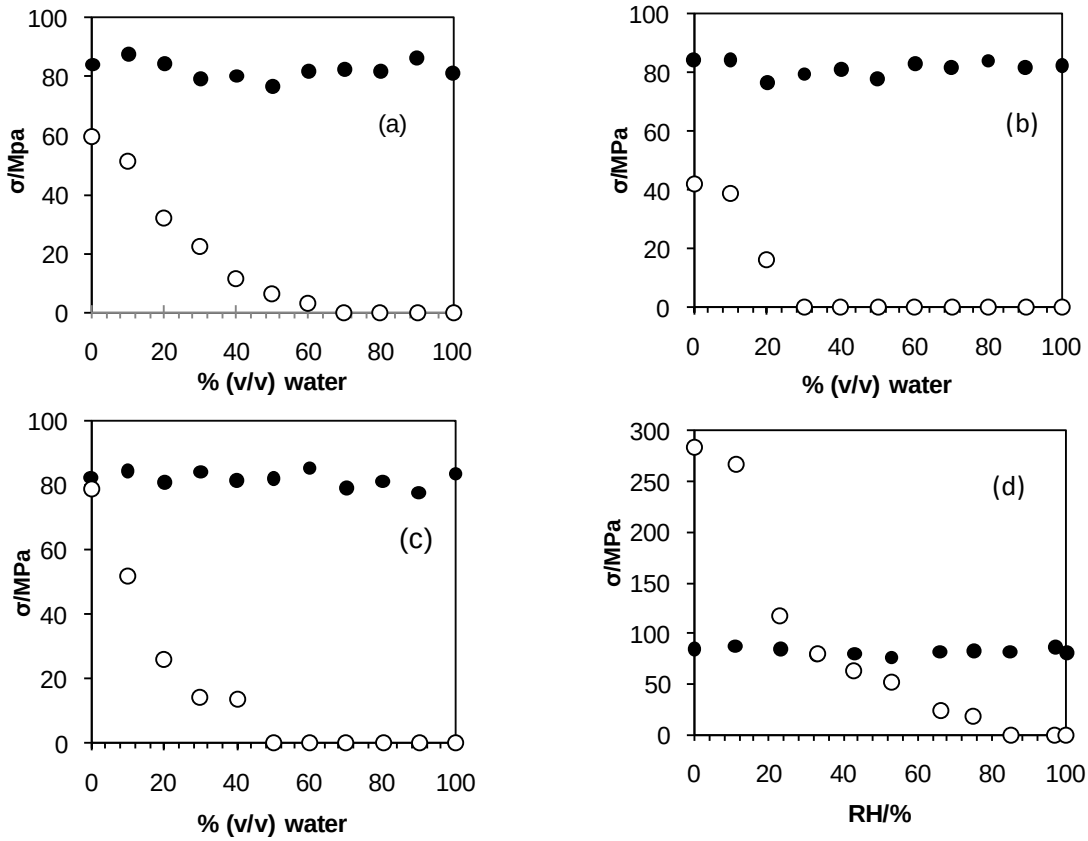

Figure 4.3 Effects of film exposure at 20 ± 1°C on the ultimate tensile strength, σ, of TPS ϕ) and APTPS (●) films: (a) glycerol/water immersion (b) ethanol/water

immersion (c) isopropanol/water immersion and (d) RH conditioning. Immersion was for 5 min and conditioning for 7 days at the specified temperature.

The tensile strength of the TPS and APTPS films after conditioning at different values of RH is shown in Figure 4.3(d). A significant difference ($p \leq 0.05$) between the σ values of the TPS and those of the APTPS films, at a given value of RH, can be seen over most of the RH range. The tensile strength of the TPS film is considerably higher than that of the APTPS film up to RH ≈ 23% but decreases to a much lower level than that of the APTPS material with a further increase in RH. Interestingly, the tensile strength of the TPS material appears to be similar to that of APTPS films at RH ≈ 33% (see Figure 4.3(d)). At RH > *ca*. 85% the integrity of the TPS film was completely destroyed and no meaningful tensile measurements could be made. Forssell *et al.* (2002) also studied the sensitivity of starch-based films to RH at ambient environment and observed a decline in the tensile strength of the films when exposed to increasing RH levels. Changong and Lumdubwong (2008) reported a significant effect of RH on the mechanical properties of thermoplastic sheets extruded from starch-based materials and Mehyar and Han (2004) observed a decrease in tensile strength of high amylose rice starch and pea starch films when the relative humidity was increased from 51% to 90%. In contrast, the APTPS film maintained its tensile strength across the entire range of RH and water content as a result of the immersion in the different mixtures studied in the present work. These results suggest that the TPS film, in contrast to the APTPS film, is sensitive to systems of high water content and/or high RH levels at which its mechanical properties are severely impaired.

The effect of water on the elongation at break, ε_b, for the TPS and the APTPS starch-based films is presented in Figures 4.4(a) to (c). The ε_b for the TPS film immersed in glycerol/water mixtures decreased with an increase in the amount of water in the mixture up to the water content of *ca*. 50% (v/v); after that, the material had no measurable elongation under the conditions of the test (see Figure 4.4(a)). The elongation of the TPS films immersed in the isopropanol/water mixtures exhibited similar behaviour to those observed in the glycerol/water mixtures (see Figure 4.4(c)). However, the TPS film immersed in the ethanol/water mixture lost its elongation at break at the lower water content of *ca*. 20% (v/v) (see Figure 4.4(b)). These observations can be explained again by the ability of ethanol and isopropanol to extract water molecules from the TPS matrix thereby rendering it brittleness. The effect of RH on ε_b for the TPS and APTPS films is shown in Figure 4.4(d). The results indicate that ε_b for the TPS film is significantly lower ($p \leq 0.05$) than that of the APTPS film at all values of RH. Furthermore, ε_b for the TPS film decreased to zero as the RH increased and the integrity of the film disappeared. The TPS film samples were found to be relatively flexible at low RH values but lost their flexibility at RH $\geq$ 75%. The results obtained in this section are consistent with those reported in Section 4.1.1 which might be attributed to the hydrophilic nature of the starch-based material. On the other hand, ε_b of the APTPS film samples did not vary significantly across the studied range of water-glycerol, water-ethanol and water-isopropanol mixtures (Figures 4.4(a) to 4.4(c)) or RH conditions (Figure 4.4(d)).

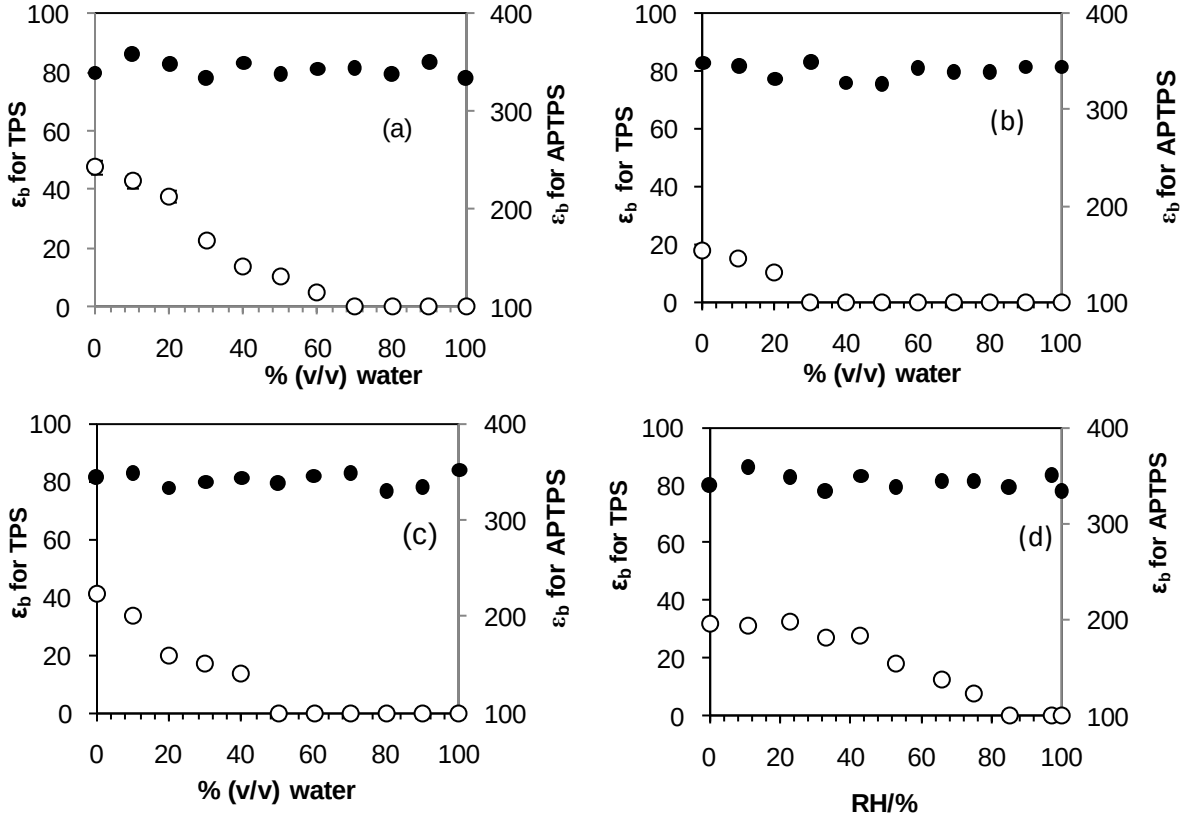

Figure 4.4 Effects of film exposure at 20 ± 1°C on the elongation at break, ε_b, of TPS (○) and APTPS (●) films: (a) glycerol/water immersion (b) ethanol/water immersion (c) isopropanol/water immersion and (d) RH conditioning. Immersion was for 5 min and conditioning for 7 days at the specified temperature.

The effect of water on the Young's modulus, *E*, of the TPS film immersed in glycerol/water mixtures is shown in Figure 4.5(a) where the value of *E* decreased with an increase in the water content. The values of *E* for the APTPS film were higher than those of the TPS film at all the water contents. In the ethanol/water and isopropanol/water mixtures, the modulus of the TPS was observed only up to *ca*. 20% and 40% (v/v) water content respectively as shown in Figures 4.5(b) and 4.5(c). The effect of RH on the value of *E* for the TPS and APTPS films is shown in Figure 4.5(d).

The values of *E* for the APTPS films are significantly ($p \leq 0.05$) higher than those of the TPS films at all the RH investigated. For values of RH > *ca*. 85%, the *E*-values for the TPS film could not be determined due to the partial dissolution of the material and subsequent loss of integrity. In contrast, the *E* values for the APTPS film remained relatively constant at all RH and this is consistent with the results obtained in the investigation of water-based mixtures. Chaléat *et al.* (2008) reported that increasing RH reduces the Young's modulus of plasticised starch and this is in agreement with the results of the present study. Stading *et al.* (2001) also observed a decrease in *E* of starch films when the RH increased from 20% to 80%.

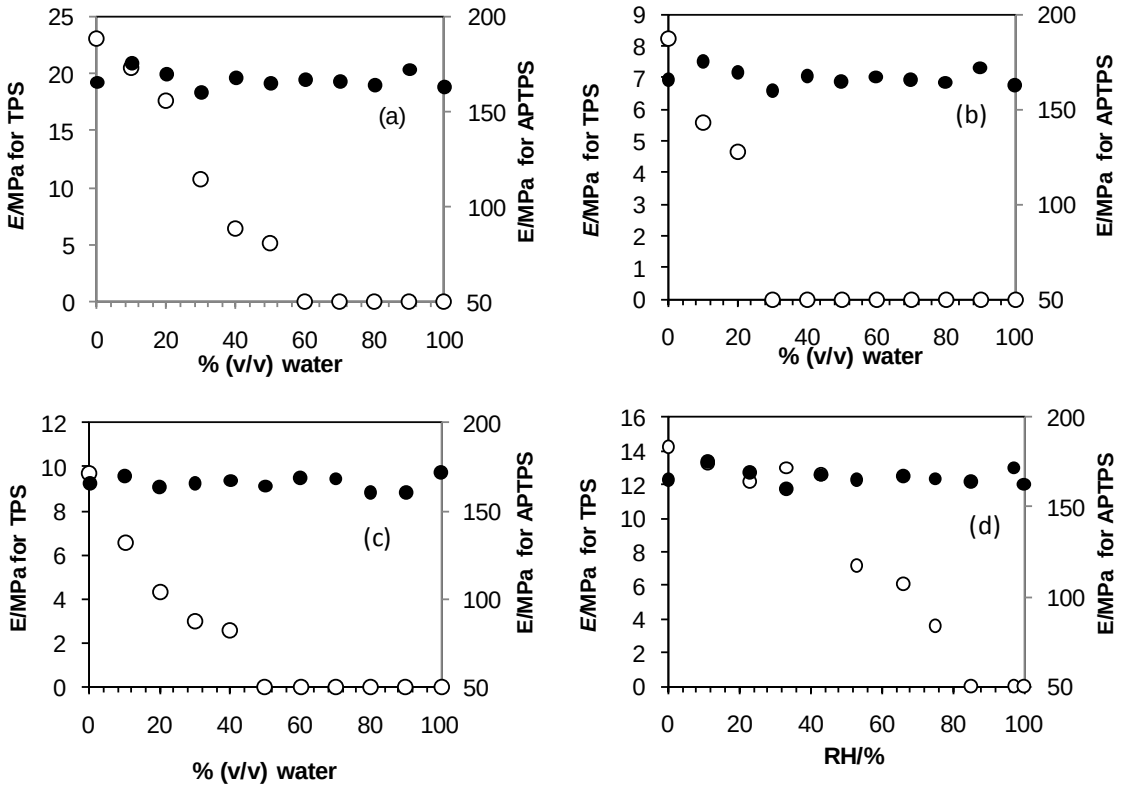

Figure 4.5 Effects of film exposure at 20 ± 1°C on the elastic modulus, E, of TPS (○) and APTPS (●) films: (a) glycerol/water immersion (b) ethanol/water immersion (c) isopropanol/water immersion and (d) RH conditioning. Immersion was for 5 min and conditioning for 7 days at the specified temperature.

The observed effect of water content in the immersing mixtures on the properties of TPS and APTPS films suggests that TPS film cannot be used for packaging of foodstuffs that have moisture content higher than *ca*. 20% (v/v) since the mechanical properties of the film are severely impaired beyond this point. However, no such a severe decrease in the mechanical properties of APTPS films was observed in any of the water-based mixtures systems investigated. It is suggested that the water content of the foodstuff should not exclude the choice of the latter as a packaging material. The results suggest that the presence of water in the solvent system has the ability to dissolve the hydrophilic matrix of the TPS material. Therefore, food compatibility of TPS films might limit its applications in the packaging of aqueous foods, acidic and/or alcoholic food products unless it is modified or blended. However, the APTPS films were observed to be mechanically stable in all of the water-based mixtures investigated.

The experiments on the effect of water on the mechanical properties of the TPS and APTPS films also revealed that the TPS film tends to dissolve in water-based mixtures that have high water contents. It therefore appears that this material is not suitable for the packaging of food products with $a_w > ca.$ 0.75 where significant mechanical strength is required since the films lost their mechanical integrity at 75% RH. Nevertheless, TPS film can be used for packaging of food products of low a_w levels. Many food products such as processed meats, bakery and hard dairy goods, some fresh produce, caramel, honey, noodles and dried fruits have a_w values ranging from 0.6 to 0.99 (Roos, 2001). Thus, some of these products have a_w values that are below that of the apparent critical limit for the TPS material in the current study. If TPS materials can be utilised effectively only for packaging of foodstuffs that have $a_w < 0.75$, then it would appear

that microbial contamination should not be of a concern in such cases since bacterial contamination is only of concern at levels of $a_w \approx 0.86 – 0.99$ (Roos, 2001).

Although bacterial contamination is only of concern at levels of $a_w \approx 0.86 – 0.99$, yeasts and moulds can contaminate food products in the intermediate moisture range of $a_w >$ *ca.* 0.65 – 0.9 (Chirife & Maria Del Buera, 1994). Therefore, these AM films can be useful at these water activity levels. In contrast, the results obtained for the APTPS film suggest that it can be applied across the entire range of a_w values for packaging of food products but consideration will have to be given to protect such products against microbial contamination from yeasts, moulds and bacteria. In agreement with the APTPS results, previous studies have shown that starch-based films can be used for packaging of food products that have higher a_w levels than those investigated in this study (García *et al.*, 1998).

4.2 Preparation of Antimicrobial Films from TPS Material

From the previous sections that investigated the suitability of the TPS material for the prospective packaging of foodstuffs, the results suggest that this material has the potential to be applied for the packaging of foodstuffs that have $a_w < 0.75$. The TPS films containing the AM agents were successfully prepared by compression moulding. The thicknesses of the AM films prepared by compression moulding were determined and the results demonstrated that the thickness varied in the range of 250-310 µm. The average thickness of the compression moulded starch-based films incorporated with linalool, carvacrol and/or thymol was found to be 300 µm, 283 µm and 280 µm respectively. There was no significant difference in the thickness of AM TPS films with

the incorporated with 2% and 4% (w/w) linalool, carvacrol or thymol. However, the incorporation of 6% (w/w) carvacrol or thymol into the TPS films resulted in a reduced thickness of TPS films. These films are very thick but their applications will depend on the type food to be packaged. Such thick films could be used as trays or liners for other types of packaging.

4.3 Determination of AM Agents Concentration in TPS Films

It was anticipated that there might be a potential loss of some of the AM agents by evaporation during processing at increased temperatures. In order to determine the residual concentration of each of the AM agents retained in the TPS films after compression moulding, actual concentrations were determined using gas chromatography. Although the loss had been reported before, they were reported here using a different packaging substrate. The concentrations of linalool, carvacrol and thymol retained in the film after compression moulding are presented in Table 4.1. The results show a significant loss of AM agent in the heat pressed starch-based film.

Table 4.1 The post processing concentration of linalool, carvacrol and thymol from heat pressed TPS film

Formulation	**Post-processing conc. % (w/w)**		
conc.% (w/w)	**Linalool**	**Cavacrol**	**Thymol**
2	0.67 ± 0.01^{a}	0.83 ± 0.02^{a}	0.86 ± 0.08^{a}
4	1.04 ± 0.06^{b}	1.12 ± 0.05^{b}	1.18 ± 0.03^{b}
6	2.91 ± 0.1^{c}	3.49 ± 0.03^{c}	3.76 ± 0.2^{c}

Mean ± standard deviation (n=3). Means in the same column for each treatment followed by different letters are significantly different ($p = 0.05$).

A GC analysis indicated that the average retention of linalool in the TPS film after heat pressing was *ca.* 36%. The retention of carvacrol in the TPS film incorporated with 6% (w/w) was determined to be *ca.* 58.2%. The results suggest that the starch-based film retained a higher agent concentration (*ca.* 62.7%) when incorporated with 6% (w/w) thymol than that of the corresponding carvacrol and linalool. The significant loss of the AM agents observed in the present study can be attributed to their high volatility when subjected to the temperature of 125°C during the heat pressing process. The data in Table 4.1 further indicates that the loss of linalool, carvacrol and thymol is most pronounced upon the addition of 4% (w/w) of all AM agents into the film formulation in all cases. The high loss of these volatile additives is also consistent with the observations made by Rupika *et al*. (2005) who reported a major loss of carvacrol (*ca*. 3.9% (w/w) final concentration) and thymol (*ca*. 2.6% (w/w) final concentration) as a result of thermal volatilisation during processing when a 5% (w/w) target concentration of AM agent was used in the LDPE film formulations. Suppakul *et al.* (2011a) also

reported a high loss of linalool and methylchavicol upon thermal processing into LDPE film.

4.4 Scanning Electron Microscopy of Starch-based Films

The scanning electron microscopy (SEM) micrographs of TPS films incorporated with 2% (w/w), 4% (w/w) and 6% (w/w) carvacrol are given in Figure 4.6(b)-4.6(c). The SEM micrograph for the control film is also presented in Figure 4.6(a) for comparison. It is clear from the control TPS film that the incorporated AM agent appears to aid in the solubility and dispersion of the starch particles as shown by the larger particles in the control TPS film (see Figure 4.6(a)). Furthermore, the addition of AM agents has affected the hydrophilicity of the starch causing it to aggregate into these larger spheres on the surface and probably throughout the film. This latter effect is more pronounced with carvacrol and thymol (see Appendix B.4) with only a minimal effect caused by linalool (see Appendix B.3) which may be due to their relative structures.

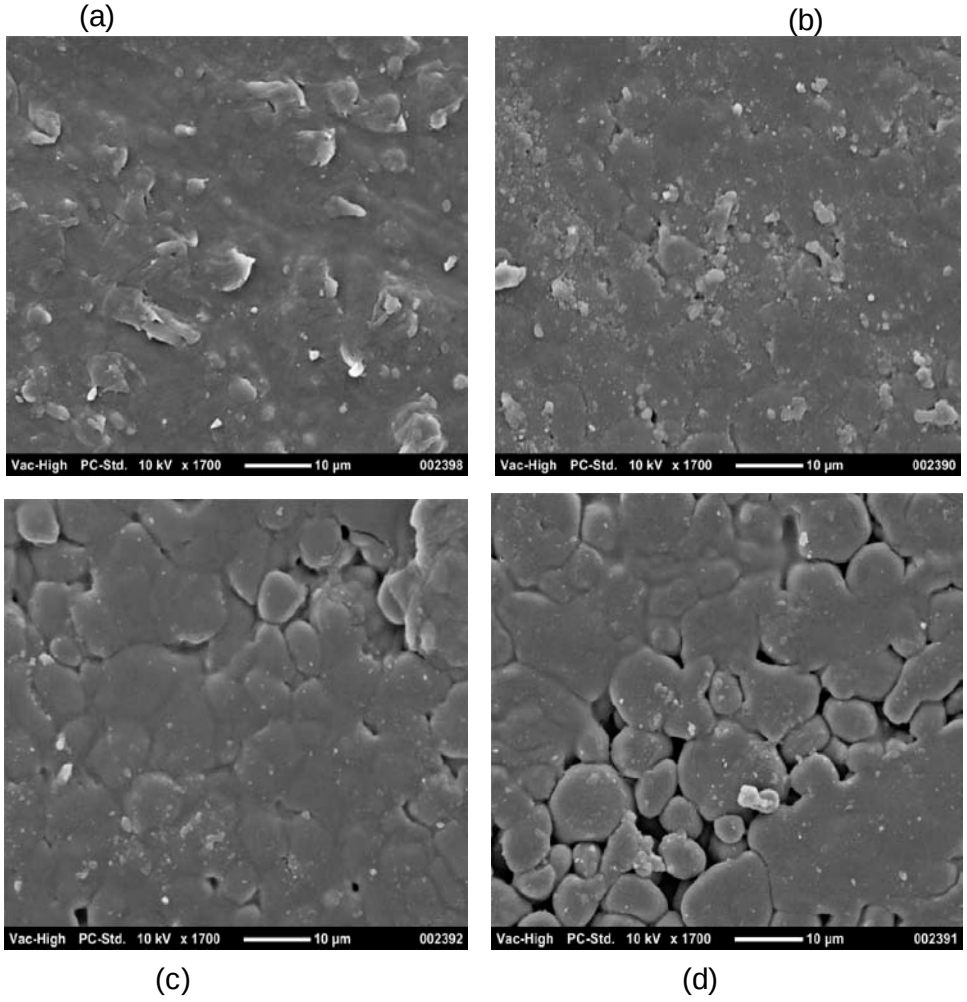

Figure 4.6 SEM micrographs of: (a) TPS film (b) TPS film containing 0.8% (w/w) carvacrol (c) TPS film containing 1.1% (w/w) carvacrol (d) TPS film containing 3.5% (w/w) carvacrol

4.5 Effects of AM Agents on Mechanical Properties of TPS Films

There is growing evidence that the physico-mechanical properties of films may be affected by relatively high concentrations of AM agents that are added to the packaging material (Han, 2003; 2005a). The effect of AM agents on the tensile strength, σ, the elongation at break, ε_b, and the elastic modulus, E, on the TPS films was explored by

analysing the stress-strain curves of the film samples. The results are summarised in Table 4.2.

Table 4.2 The tensile strength, elongation at break and Young's modulus for TPS films containing the AM agents linalool, carvacrol or thymol.

AM Agents	Conc.	Tensile Strength	Elongation at break	Young's Modulus
	% w/w	MPa	%	MPa
Control	0	119 ± 25^{a}	42.9 ± 0.2^{a}	21 ± 11^{a}
Linalool	0.67	113 ± 25^{a}	43.6 ± 0.2^{a}	21 ± 8^{a}
	1.04	108 ± 22^{ab}	45.7 ± 0.2^{ab}	22 ± 10^{a}
	2.91	94 ± 22^{bc}	48.1 ± 0.2^{ab}	24 ± 8^{ab}
Carvacrol	0.83	111 ± 28^{a}	43.2 ± 0.2^{a}	21 ± 8^{a}
	1.12	105 ± 24^{ab}	46.5 ± 0.1^{ab}	23 ± 3^{a}
	3.49	88 ± 7^{bc}	52.0 ± 0.1^{bc}	25 ± 5^{ab}
Thymol	0.86	108 ± 29^{ab}	44.0 ± 0.1^{a}	22 ± 1^{a}
	1.18	97 ± 12^{bc}	47.7 ± 0.1^{ab}	23 ± 3^{a}
	3.76	83 ± 9^{cd}	54.0 ± 0.1^{bc}	26 ± 4^{ab}

Mean ± standard deviation (n=5). Means in the same column for each treatment followed by different letters are significantly different ($p = 0.05$).

The results in Table 4.2 indicate that increasing the level of the three studied AM agents: linalool, carvacrol, or thymol in the TPS films results in a decreasing tensile strength compared with the control film. However, an average error of 20% was associated with the tensile strength suggesting the effect may not be significant in all

cases. The greatest reduction in tensile strength for the TPS film compared with the control sample was observed in the films containing 3.8% (w/w) thymol. In this case the reduction was significant ($p \leq 0.05$). Similarly, the concentration of 3.5% (w/w) carvacrol produced a seemingly significant decrease in the tensile strength of the TPS film. The effect of carvacrol addition observed in the present study is in agreement with the findings of Du *et al.* (2008a) who found that an addition of 1-1.5% (w/w) carvacrol into tomato-based films resulted in a significant reduction (from 11.4 to 8.9 MPa) in the tensile strength of the films. Zivanovic *et al*. (2005) also observed a significant decrease in the tensile strength of chitosan films when impregnated with oregano essential oils. This result is consistent with our findings. The results of the present study are also in agreement with those of Corrales *et al*. (2009) who reported a decrease in the tensile strength of pea starch films impregnated with a grape seed extract as well as with those of Maizura *et al.* (2007) who reported a reduction in the tensile strength of starch-alginate film impregnated with 0-0.4% (v/w) lemongrass oil. The decrease in the tensile strength of the films may be caused by the interactions between the AM additives and the TPS matrix affecting the intermolecular forces within the matrix similarly to water acting as a plasticiser (Kramer, 2009)

The data for the effect of linalool, carvacrol and thymol on the elongation at break, ε_b, for the AM starch-based films is also presented in Table 4.2. The results suggest that an increase in the concentration of the AM agents tends to increase slightly the elongation at break. A statistical analysis of the results suggests that at the concentrations of 0.7% (w/w) linalool or 0.8% (w/w) carvacrol, the effect of linalool or carvacrol addition on the elongation at break is insignificant and that the effect of 0.9% (w/w) thymol is

arguably insignificant. Nonetheless, a significant ($p \leq 0.05$) increase in the elongation at break of up to *ca*. 10% is apparent at 2.9% (w/w) linalool, 3.5% (w/w) carvacrol or 3.8% (w/w) thymol in all cases. These results are in agreement with the work of Pelissari *et al*. (2009) who reported that the addition of oregano essential oils (EOs) at varying concentration into starch-chitosan films increases the elongation at break. Furthermore, Zinoviadou *et al*. (2010) reported that the addition of EOs to whey protein isolate films increases the elongation at break when the concentration is increased from 0.5 to 1.5% (w/w). The observed increase in the elongation of the AM films might be attributed to linalool, carvacrol or thymol acting as plasticisers in the material. This increase in the elongation at break of the AM films assumed to have been caused by AM agents is consistent with the previous studies conducted by Ozdemir and Floros (2008a), Kristo *et al.* (2008) and Imran *et al*. (2010).

The effect of the AM agents on the modulus of elasticity, *E*, of the starch-based films was also explored. The data in Table 4.2 suggest that the incorporation of the three AM agents into the films at the different concentrations tested causes a significant increase in the Young's modulus in all cases. The data further suggest that the increase in Young's modulus is most pronounced at the concentration of 3.8% (w/w) thymol in the film. In this case an increase of *ca*. 11% in the modulus was observed compared to the control sample. At the concentration of 2.9% (w/w) linalool or 3.5% (w/w) carvacrol, an increase of *ca*. 9% and 7% in the Young's modulus compared to the control were observed for carvacrol and linalool respectively. The results of the present study are not in agreement with the results of Altiok *et al*. (2010) who reported a decrease in the Young's modulus of chitosan films impregnated with 0.2-1.2% (v/v) thymol oil.

4.6 Effects of AM Agents on Water Vapour Permeability of Films

The water vapour permeability (WVP) is one of the key factors to be considered when selecting biobased materials for food packaging. Therefore, the effect of various concentrations of linalool, carvacrol and thymol incorporated into the TPS film on their WVP was investigated. The averaged numerical values demonstrating the effects of linalool, carvacrol and thymol on the WVP of starch-based films are presented in Table 4.3.

Table 4.3 WVP values of starch-based films containing the AM agents: linalool, carvacrol and thymol.

AM Agent	Conc. % w/w	WVP × 10^{-2} g m^{-1} day^{-1} kPa^{-1}
Control	0	10.1 ± 0.001^{a}
Linalool	0.67	10.3 ± 0.002^{a}
	1.04	10.6 ± 0.005^{a}
	2.91	11.5 ± 0.005^{ab}
Carvacrol	0.83	10.4 ± 0.002^{a}
	1.12	10.6 ± 0.001^{a}
	3.49	11.2 ± 0.005^{ab}
Thymol	0.86	10.4 ± 0.001^{a}
	1.18	10.7 ± 0.001^{ab}
	3.76	13.2 ± 0.007^{bc}

Mean ± standard deviation (n=3). Treatment with same letter within row is not statistically significant difference ($p \leq 0.05$).

The data given in Table 4.3 show that at low AM agents concentrations, the WVP values were not significantly different ($p > 0.05$) from the control. However, at the concentrations of 2.9% (w/w) linalool or 3.5% (w/w) carvacrol in the TPS film the WVP of the films increased significantly ($p \leq 0.05$) compared to the control. The greatest effect of carvacrol on the WVP was observed for starch-based films containing 3.5% (w/w) of this additive which is consistent with the findings of Pruneda *et al*. (2008) who reported an increase in the water permeability of soy protein isolate films incorporated with Mexican oregano. The increases of WVP values of TPS films may be due to the effect of AM agents as they affect the hydrophilicity of the starch by causing it to aggregate into these larger spheres (see Figure 4.5). The results of the present study are also in agreement with those of Maizura *et al.* (2007) who observed an increase of WVP values of starch-alginate film impregnated with 0.2% (v/w) lemongrass oil. Furthermore, the results of the present study are in agreement with those of Rojas-Grau *et al*. (2007) who found that 0.1% (w/w) carvacrol incorporated into alginate-apple puree films did not affect the WVP of the resultant films. Lim *et al*. (2010a) reported that 0.4-1.0% (w/v) carvacrol actually reduced the WVP of *Gelidium corneum* film.

Generally, the inclusion of a plasticiser into polymeric materials such as glycerol and water in case of the present study increases the WVP of the resultant films as suggested by Dangaran *et al.* (2009) and Mali *et al*. (2004). Such an increase of WVP as a result of the incorporation of AM agents may be attributed to the structural modifications of the starch network occurring when AM agents (linalool, carvacrol and thymol) are added. The addition of comparatively small molecular species such as AM molecules to

the polymer matrix may lower the crystallinity and facilitate a higher molecular transport through the matrix.

4.7 Effects of AM Agents on Thermal Properties of TPS Films

The differential scanning calorimetry (DSC) analysis of the TPS impregnated with the AM agents demonstrated a wide variation in the crystalline melting temperature (T_m) in the three systems derived from the corresponding enthalpy of melting (ΔH). A DSC thermogram of TPS films incorporated with 2.9% (w/w) linalool, 3.5% (w/w) carvacrol and 3.8% (w/w) thymol is presented in Figure 4.7. The average values of the T_m and ΔH for each of the AM systems is presented in Table 4.4.

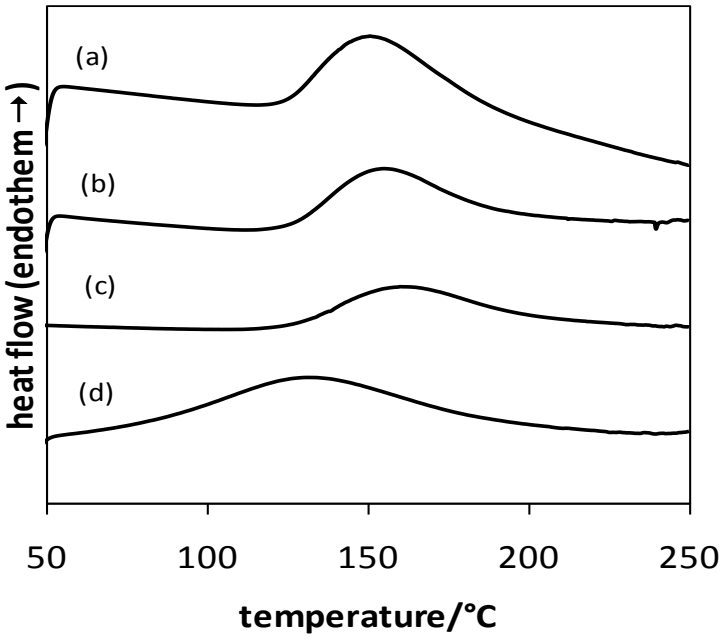

Figure 4.7 DSC thermograms of: (a) control TPS film and (b) TPS film containing 3.8% (w/w) thymol (c) TPS film containing 3.5% (w/w) carvacrol (d) TPS film containing 2.9% (w/w) linalool

Table 4.4 The T_m and ΔH for starch-based films containing linalool, carvacrol or thymol

AM agent	Conc. % (w/w)	T_m / °C	ΔH / J g^{-1}
Control	0	150.9 ± 0.1[a]	188.7 ± 0.6[a]
Linalool	0.67	142.7 ± 2.0[ab]	187.3 ± 3.8[a]
	1.04	141.5 ± 4.5[ab]	186.9 ± 0.5[a]
	2.91	141.0 ± 0.5[ab]	181.1 ± 2.0[ab]
Carvacrol	0.83	147.5 ± 1.7[a]	186.5 ± 4.1[a]
	1.12	146.0 ± 0.1[a]	186.6 ± 2.9[a]
	3.49	145.3 ± 4.2[a]	184.3 ± 2.8[ab]
Thymol	0.86	150.7 ± 2.2[a]	188.1 ± 5.7[a]
	1.18	148.2 ± 2.3[a]	186.5 ± 4.9[a]
	3.76	146.5 ± 1.8[ab]	185.7 ± 6.1[a]

Mean ± standard deviation (n=3). Treatment with same letter within row is not statistically significant difference (p = 0.05).

The DSC analysis of the control film showed an endothermic peak (Figure 4.7) with an average T_m of *ca.* 150.9°C. Analysis of the thermograms obtained at low concentrations of the AM agents did not produce a significant effect on the crystallinity of the material. However, the concentrations of 2.9% (w/w) linalool 3.5% (w/w) carvacrol and 3.8% (w/w) thymol in the formulation caused a decrease in the T_m compared to the control TPS film (see Figure 4.7). The results suggest that the addition of linalool, carvacrol or thymol into the TPS film decreases the intermolecular interaction within the film that, in turn, causes the observed decrease in the degree of crystallinity. The decrease in the

latter is responsible for changes in the various physico-mechanical and transport properties as well as in the water uptake reported above (see Section 4.1.1). According to Garcia *et al*. (2009), the addition of additives into a polymeric material may interfere with chain association and cause a possible decrease in the film crystallinity.

4.8 Effects of Antimicrobial Agents on Transparency of TPS Film

The effect of linalool, carvacrol or thymol on the transparency of the TPS films was determined using a UV -Visible spectrophotometer. The transparency of the TPS films was determined by measuring the percentage transmittance of light at a wavelength of 600 nm (T_{600}). The transparency of the starch-based films incorporated with linalool, carvacrol or thymol at three different concentrations is shown in Figure 4.8.

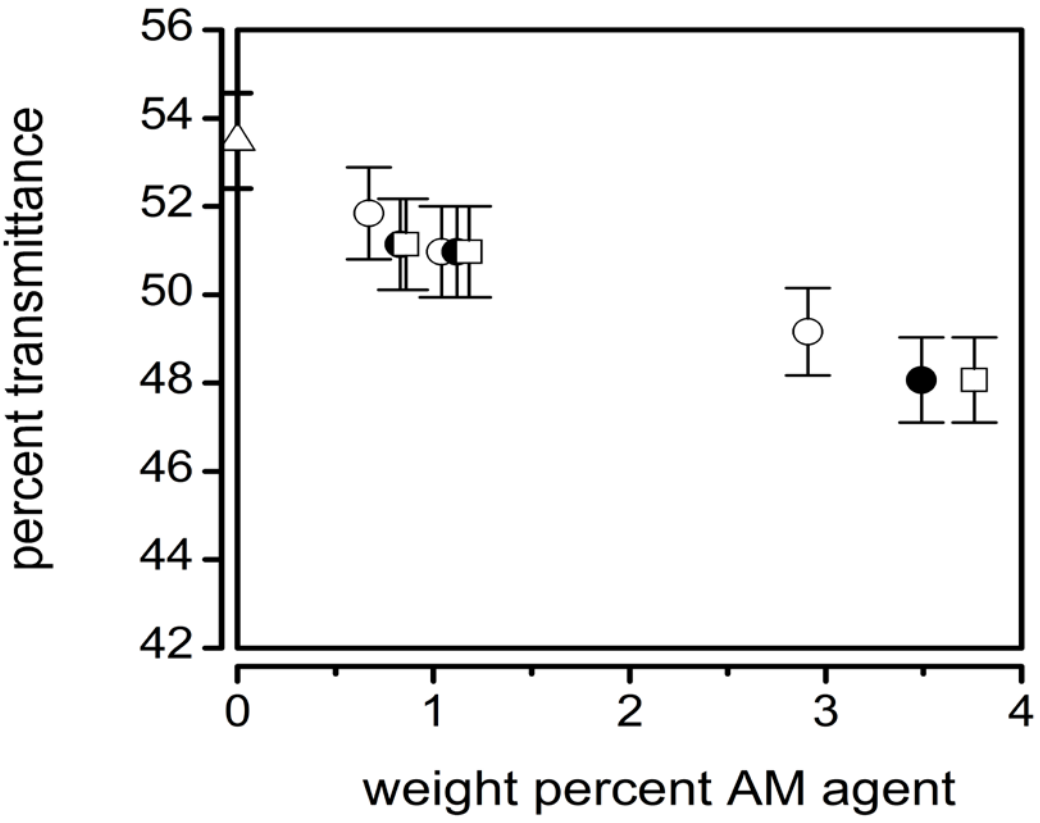

Figure 4.8 Transmittance at 600 nm of: (a) control TPS film and (b) TPS films containing 0.7%, 1.0% or 2.9% (w/w) linalool, 0.8%, 1.1% or 3.5% (w/w) carvacrol, or 0.9%, 1.2% or 3.8% (w/w) thymol.

There was no measureable variation in the light transmission at 600 nm of the films upon the incorporation of 0.7% (w/w) or 1.0% (w/w) linalool, 0.8% (w/w) or 1.1% (w/w) carvacrol or 0.9% (w/w) or 1.2% (w/w) thymol, compared with the control film. However, the concentration of 2.91% (w/w) linalool, 3.5% (w/w) carvacrol, or 3.8% (w/w) thymol slightly lowered the light transmission, suggesting that incorporation of AM agents at levels >6% (w/w) during film formulation may adversely affect the transparency of the TPS film. Significant transmission of light by the films was

observed in the visible region of 400-800 nm that makes these formulations suitable candidates for use as clear packaging films. In the wavelength range of 400-800 nm, all the AM films transmitted light at this visible region that makes these formulations suitable candidates for use as clear packaging films. The results in the present study are consistent with those of Rao *et al*. (2010) who investigated the effect of AM agents on the mechanical properties of chitosan and guar gum composite films. The opacity of packaging materials derived from natural sources to short-wavelength light has been reported by Fang *et al*. (2002) who investigated also the tensile and barrier properties of whey protein isolate films. The observed opacity to short-wavelength (UV range of 195-280 nm) of the starch-based materials in the present study is significant suggesting that these materials can be used to protect food products from deterioration caused by light as also suggested by Rao *et al*. (2010).

4.9 The Coating of Antimicrobial Films

Coating of AM agents onto packaging materials is one of the successfully used methods, in the past to produce AM films (Rardniyom, 2008a). Therefore, in this section the APTPS films were coated with an inert medium containing linalool, carvacrol and thymol at three different concentration levels. Due to the volatility of the AM agents used in this study, it was expected that AM agents might be lost by evaporation from the surface of the coating substrate during the drying of the film. However, the coating process was conducted at room temperature in order to minimise the loss of the AM agents. Therefore, target concentrations of 1%, 3% and 5% (w/w) for all three AM agents were incorporated so as to identify the optimal concentration of each of the AM agents for future AM activity studies. The average thickness of the

starch-based films coated with linalool, carvacrol and/or thymol was found to be 185 µm, 131 and 164 µm respectively. The residual concentrations of linalool, carvacrol or thymol retained in the MC-HPMC coatings are presented in Table 4.5. The results show a significant retention of AM agent in the coated starch-based films.

Table 4.5 The post processing concentration of linalool, carvacrol and thymol from MC-HMPC coated starch-based film

Formulation	**Post-processing conc. % (w/w)**		
conc.% (w/w)	**Linalool**	**Carvacrol**	**Thymol**
1	0.48 ± 0.01^{a}	0.48 ± 0.01^{a}	0.48 ± 0.01^{a}
3	1.43 ± 0.03^{b}	1.43 ± 0.03^{b}	1.43 ± 0.03^{b}
5	2.38 ± 0.05^{c}	2.38 ± 0.05^{c}	2.38 ± 0.05^{c}

Mean ± standard deviation (n=3). Means of each treatment in a column with different superscript letter are statistically significant difference (p = 0.05).

In the starch-based films coated with MC-HPMC, the residual linalool, carvacrol or thymol concentrations in the coatings of the dried films were close to the respective formulation concentrations of 1% (w/w) with an average retention of *ca*. 95%. Therefore, the respective average concentration of each of these AM agents, on the basis of the total weight of the dry film, was 0.48% (w/w) in each case. The retentions of linalool, carvacrol or thymol coated onto the starch-based films at 3% (w/w) and 5% (w/w) were also found to be significantly high (see Table 4.5). The respective concentration of AM agent on the basis of the total weight of the dry film was found to be 1.4% (w/w) and 2.4% (w/w) in each case. Again, the high retention of AM agent in

the coatings can be attributed to the low temperature used during the coating process. The significant retention of AM agents coated onto the starch-based films in the present study are consistent with the results obtained by Rardniyom (2008a) who reported considerable retention (96.2%) of carvacrol in ethylacrylate-methylmethacrylate coatings.

4.10 Migration of Antimicrobial Agents from Starch-Based Films

The results shown in the above sections (see sections 4.2 and 4.8) suggest that AM films can be prepared from both TPS and APTPS starch-based films. The AM films prepared by compression moulding or by coating retained a sufficient amount of AM agent as shown in Table 4.1 and Table 4.5 for TPS films and APTPS films respectively. The volatile AM agent retained in the film must be released from the film onto the food surfaces in order to control microorganisms that can potentially deteriorate the food. Therefore, the release of AM agents from the films was investigated using food simulants. Immersion of the starch-based films or coated films did not adversely affect the integrity of the materials.

4.10.1 Release of Antimicrobial Agents into Food Simulants

The migration into isooctane (a fatty food simulant) of the AM agents from the starch-based films prepared by compression moulding or from the MC-HMPC coatings was studied at three different temperatures (15, 25 and 35°C). Figure 4.9(a) shows the plots of mass fraction (m_t/m_∞) of carvacrol released from the compression moulded films into the simulant versus time at the three temperatures. The migration of carvacrol from the

MC-HPMC coated samples into isooctane is shown in Figure 4.9(b). Similar behaviour was observed for linalool and/or thymol migration into isooctane at these temperatures for the compression moulded and the MC-HPMC coated films (Appendix C.1 and C. 2). From Figure 4.9(a) and (b), it can be seen that carvacrol is readily released into isooctane from both film forms.

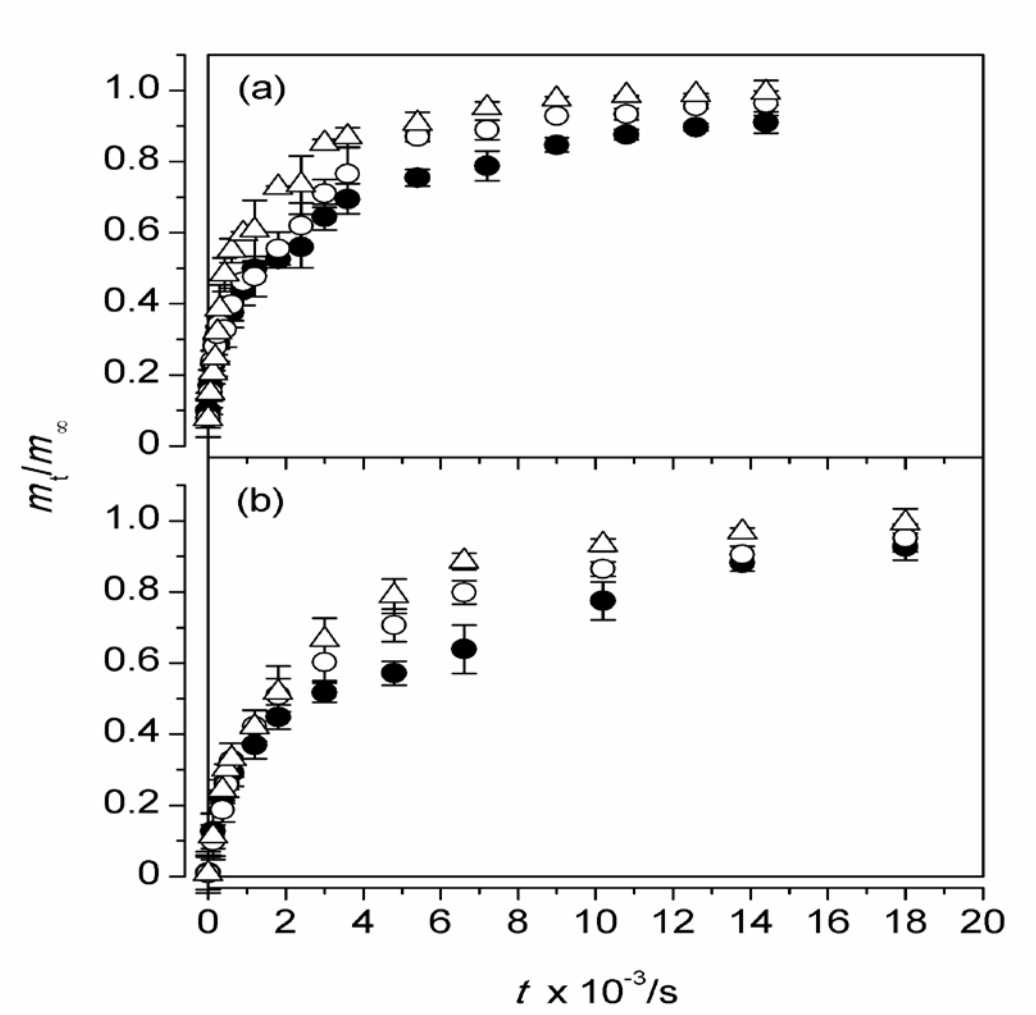

Figure 4.9 Plot of the mass fraction m_t/m_∞ of carvacrol released into isooctane versus t at (●) 15, (○) 25 and (Δ) 35 °C from: (a) heat pressed TPS and (b) MC-HPMC coated starch-based film.

It is evident from Figure 4.9(a) that the higher the temperature, the faster is the migration rate of carvacrol, as could have been anticipated. At the lowest temperature of 15°C, the release of carvacrol into isooctane reached equilibrium within *ca.* 9000 s. For thymol and linalool at this temperature equilibrium was achieved within *ca*. 7200 s (Appendix C.1 and C. 2). Increasing the temperature to 35°C increased the release rate of carvacrol and equilibrium was attained within *ca.* 7200 s (see Figure 4.9(a)). In the compression moulded films containing thymol or linalool similar migration profiles were demonstrated and the time to reach equilibrium at 35°C was *ca*. 5400 s for both AM agents. The increased release rate of the AM agents from the starch-based films at the higher temperatures is attributed to the enhanced mobility of the AM molecules at the elevated temperatures (Zhu *et al.*, 2006). From Figure 4.9(b) it can be seen that the release of carvacrol from the MC-HPMC coatings also increases with the increase in temperature as again could have been anticipated. Similar trends were obtained for the release of thymol and linalool into isooctane at 15, 25 and 35°C (Appendix C.1 and C. 2).

The release data for the AM agents in the compression moulded and MC-HPMC coated films shown in Figure 4.9 were further analysed in terms of an overall kinetic model and a diffusion model. The overall kinetic analysis plots for the release of carvacrol from the compression moulded and the MC-HPMC coated films at 15, 25 and 35°C, are shown in Figures 4.10(a) and 4.10(b) respectively. In all cases the data fit an overall kinetic model with an expected increase in the release rate with increasing temperature. Similar trends were observed for the migration of thymol and linalool from their respective substrate films at these temperatures (Appendix C.3 and C. 4). The initial release rate, v_0

and the overall rate constant for release, k_1 that were obtained from the analysis of the data by the kinetic model for the two kinds of films are presented in the Table 4.6.

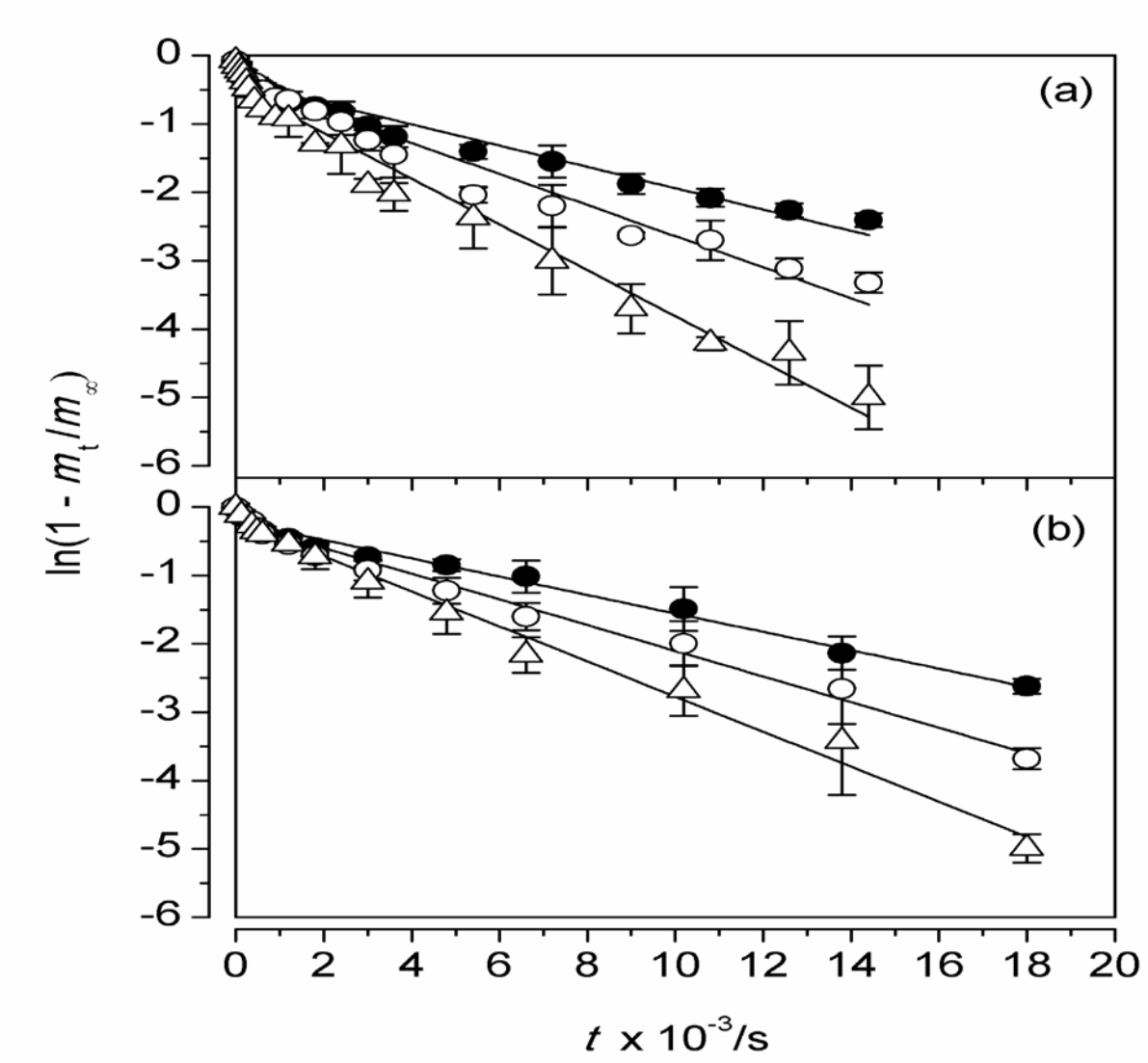

Figure 4.10 Plots of $\ln(1 - m_t/m_\infty)$ versus t for the migration of carvacrol into isooctane at (●) 15, (○) 25 and (Δ) 35 °C from: (a) heat pressed and (b) MC-HPMC coated starch-based film.

Table 4.6 The overall kinetic and the diffusion anlayses for the release of carvacrol, thymol and linalool from: (a) heat pressed and (b) MC-HMPC coated starch-based film into isooctane at 15, 25 and 35°C.

AM Agent	Temperature /°C	Kinetic Analysis		Diffusion Analysis	
		$v_0 \times 10^{-4}$ /g s^{-1}	$k_1 \times 10^{-4}$ /s^{-1}	$D \times 10^{-13}$ /m^2 s^{-1}	$k_2 \times 10^{-4}$ /s^{-1}
(a) Release from Compression Moulded Starch-Based Film					
Linalool	15	0.1	1.8	9.5	1.1
	25	0.2	2.3	13.0	1.7
	35	0.4	3.2	19.0	2.4
Carvacrol	15	0.2	2.0	6.3	1.2
	25	0.3	2.7	7.9	1.9
	35	0.5	3.6	12.9	3.0
Thymol	15	0.1	1.8	12.0	0.9
	25	0.2	2.4	21.1	1.7
	35	0.6	3.5	29.7	2.8
(b) Release from MC-HMPC Coating of Starch-Based Film					
Linalool	15	0.3	2.8	5.1	2.5
	25	0.4	3.1	6.3	2.5
	35	0.7	3.6	9.4	4.8
Carvacrol	15	1.2	1.5	2.2	1.5
	25	1.8	2.8	3.9	1.8
	35	3.6	3.8	8.7	2.4
Thymol	15	0.8	1.7	2.7	1.6
	25	1.2	2.2	3.0	2.1
	35	3.1	3.4	6.1	2.8

The results shown in Table 4.6 along with the plots in Figure 4.10 demonstrate that an overall first-order kinetic model adequately describes the release of the three AM agents into isooctane from the starch-based systems. In the case of both kinds of film, the initial release rate and the overall rate constant consistently increased with the increase in temperature from 15°C to 35°C. This observation is consistent with that of Han and Floros (1997) who have stated that an increase in temperature has a significant effect on the migration of AM agents from films.

The data from the kinetic model show an average increase in the initial release rate of about 310% and an average increase in the rate constant of about 200% for all samples over the temperature range studied in the present work. These increases are somewhat lower than what could have been expected from the rule of thumb that the rate of a chemical or physical process doubles approximately every 10°C rise in temperature. This deviation from the expected release behaviour may be due to hydrogen bonding effects between the AM agents and the different polymer matrices. As one would expect, the rate constant for the migration of linalool, carvacrol and thymol from the MC-HPMC coatings would be higher than those obtained for the compression moulded samples. This observation may be attributed to the differences in the concentration and different locations of AM agents in the two kinds of film matrices. The experimental results were also analysed by the diffusion model of migration. To apply this model, the migration of the AM agents into the food simulant from the two kinds of films was considered in two domains: the short-term and the long-term migration (Crank, 1975; Miltz, 1987).

Figures 4.11(a) and 4.11(b) show plots of m_t/m_∞ versus $t^{½}$ for the short-term release of carvacrol at 25°C from the compression moulded starch-based film and of ln(1 - m_t/m_∞) versus t for the long-term release respectively. Similar behaviour to that depicted in Figure 4.11 was also observed for the release of carvacrol into isooctane at 15 and 35°C. Similar results to those shown in Figure 4.11 were found for the compression moulded starch-based films containing thymol or linalool (Appendix C.3 and C. 4). The linearity of the plots at m_t/m_∞ < 0.6 with respect to $t^{½}$ demonstrates that the data are well described by the diffusion model given in equation 3 for the short-term migration of the AM agent. In the long-term migration of carvacrol from the moulded starch-based films, the linearity of ln(1 - m_t/m_∞) versus time (for the long-term migration (m_t/m_∞ > 0.6), according to equations 4 and 5) is also very good with correlation coefficients of r^2 = 0.991, 0.963 and 0.985 for 15, 25 and 35°C respectively.

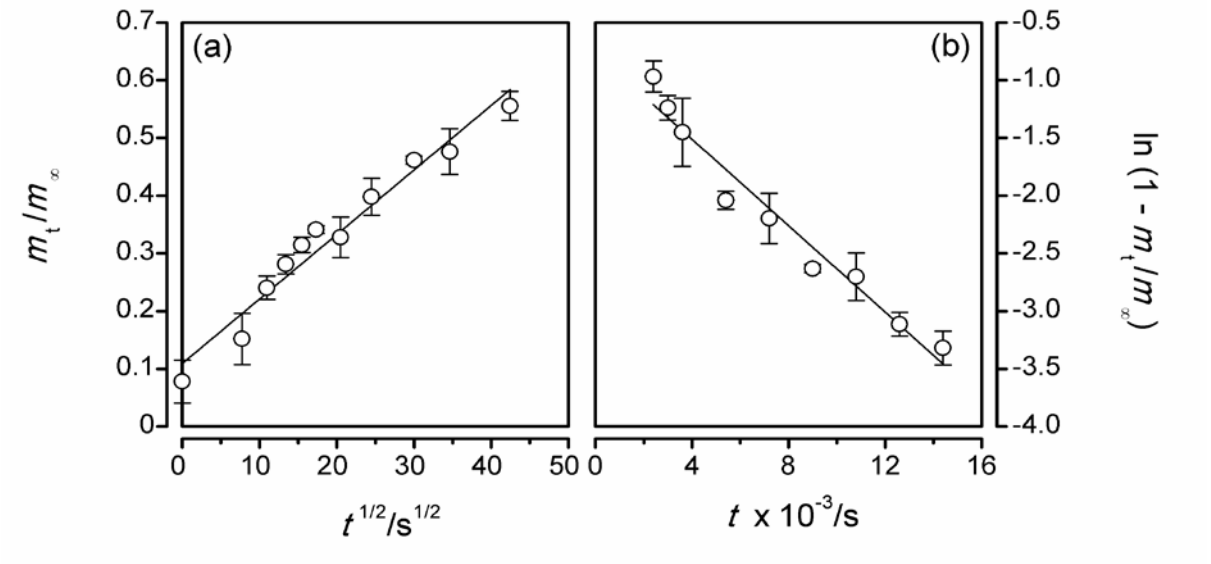

Figure 4.11 Plots of: (a) m_t/m_∞ versus $t^{½}$ and (b) ln(1 - m_t/m_∞) versus t for the migration of carvacrol from heat pressed starch-based film into isooctane at 25°C.

Figure 4.12 shows the short-term and long-term analyses of the migration of carvacrol from the coated films. The respective behaviour of these systems is similar to those shown in Figure 4.11 with good linear correlations in both time regimes. The results obtained for carvacrol and thymol systems confirm that the rate of AM agent release increases with temperature in the range of 15 to 35°C as could have been anticipated and are in agreement with the migration pattern found by Mistry (2006) for LDPE films incorporated with linalool or carvacrol. The complete numerical results of the analyses depicted in Figures 4.11 and 4.12 are also included in Table 4.6 for direct comparison.

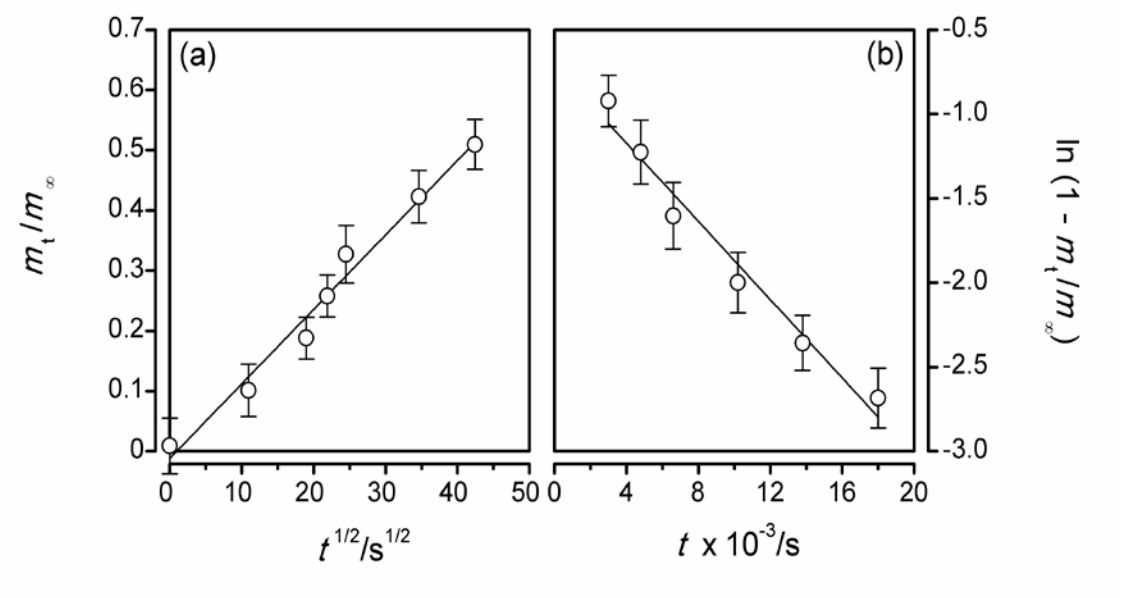

Figure 4.12 Plots of: (a) m_t/m_∞ versus $t^{½}$ and (b) $\ln(1 - m_t/m_\infty)$ versus t for the migration of carvacrol from MC-HPMC coatings on starch-based films into isooctane at 25°C.

The plot of ln(1 - m_t/m_∞) versus time for the migration of carvacrol at 25°C from the compression moulded starch-based film into isooctane yielded a straight line (r^2 = 0.963) as shown in Figure 4.11(b). From the slope of this line the diffusion coefficient, *D*, was determined. The diffusion coefficients determined from the gradients of similar regression lines shown in Figures 4.11(b) and 4.12(b) are presented in Table 4.6 for all studied systems. The results listed in Table 4.6 confirm that the diffusion coefficients of carvacrol, thymol and linalool in the compression moulded as well as in the MC-HPMC coated films increased with increasing temperature.

The effect of temperature on the diffusion coefficient for the migration of carvacrol into isooctane is plotted in Figure 4.13 according to the Arrhenius relationship given in equation 6. Similar plots (see Appendix C.11 and C. 12) were obtained for the two other AM agents. From the slopes of these plots values of the activation energy for the diffusion process, E_a, were calculated**.** The activation energy represents the sensitivity of the diffusion coefficient to temperature (Chung *et al.*, 2001b).

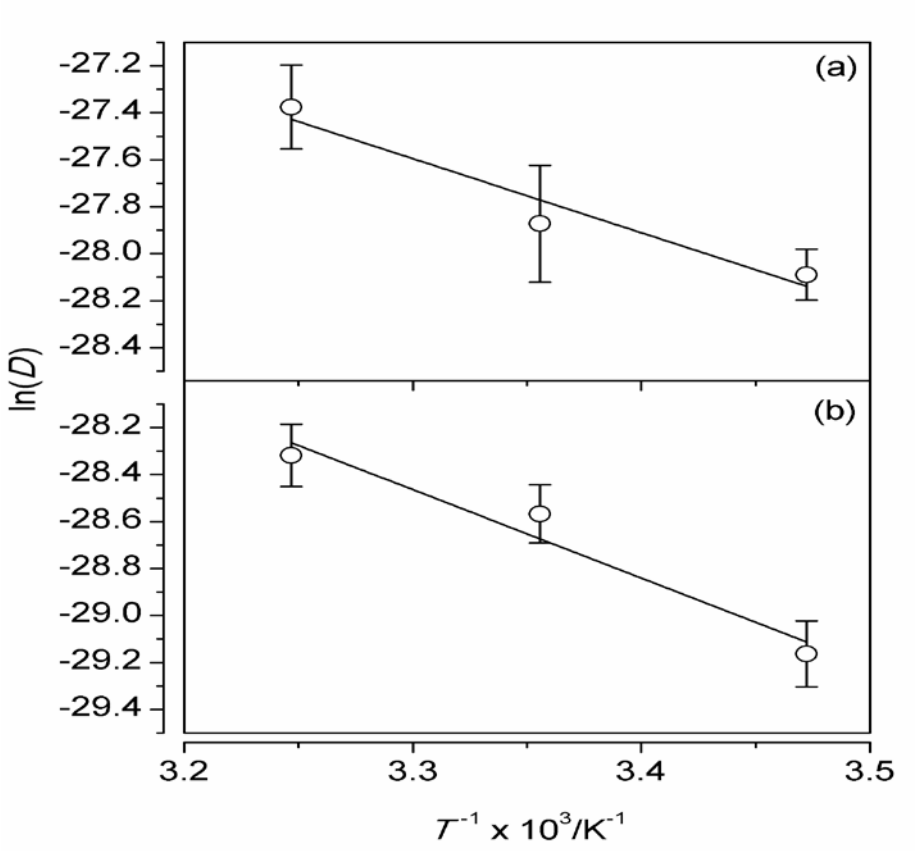

Figure 4.13 Arrhenius plots of ln(D) versus 1/T for the release of carvacrol into isooctane from: (a) the heat pressed and (b) MC-HPMC coated starch-based films.

The activation energies for the migration of linalool, carvacrol and thymol from the compression-moulded systems were found to be: 25.5, 26.2 and 33.6 kJ mol^{-1} and for the MC-HMPC coated systems: 22.5, 31.3 and 29.9 kJ mol^{-1} respectively. It can be seen that there is a clear difference between the E_a values of the AM agents in the compression-moulded films compared with the MC-HPMC coated films. In the compression-moulded films, thymol and linalool exhibited higher E_a values than in the coated films whereas the reverse was found for carvacrol. These observations presumably reflect the differences in the molecular interactions and hydrogen bonding

that exists amongst the different AM agents and the polymeric matrices. The observed differences may also stem from the different concentrations of AM agents in the moulded starch-based films and in the MC-HPMC coatings. According to Cho *et al*. (2005), a high concentration of AM agent in a polymer matrix may reduce the activation energy for diffusion due to lower molecular movements.

4.10.2 Release of Antimicrobial Agents into Atmosphere

The AM agents incorporated into or coated onto a packaging film may be lost into the atmosphere or surroundings as a function of time and temperature (Rardniyom, 2008a; Rupika, 2008a; Suppakul *et al.*, 2011a). Many studies have investigated the migration of AM agents from the packaging film into food simulants. However, very few studies have investigated the release of AM agents in the packaging films into the atmosphere. A better understanding of the release of AM agents into the atmosphere under different storage conditions is very important for successful commercial applications of AM packaging films for food packaging. To this end, the release of carvacrol from the coatings was investigated under two different storage conditions (unwrapped and wrapped with aluminium foil). The FTIR was used to monitor the loss of carvacrol from the MC-HPMC coatings. The concentration of carvacrol remaining in the MC-HPMC coatings after 28 days of storage is presented in Figure 4.14.

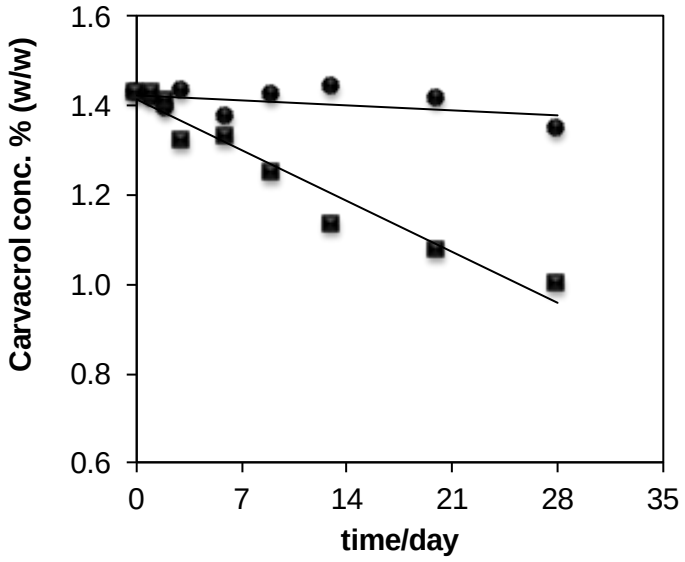

Figure 4.14 Plot of the mass of carvacrol released into atmosphere at (■) unwrapped film (●) wrapped with aluminium foil from MC-HPMC coated starch-based film.

The AM starch-based films containing carvacrol stored in the open environment without wrapping show a major loss of active agent, as expected, with retention of *ca.* 69.9% after 28 days. The results suggest that a rapid loss of carvacrol from MC-HPMC coatings happens during the first 14 days of storage. The AM films left open at ambient conditions attained equilibrium after *ca*. 21 d of storage. For the wrapped, with aluminium foil, samples that were stored at ambient conditions, the loss of carvacrol was much less by *ca.*25% after 28 days than in the sample left in the open environment. Overall, the results showed that *ca.* 94.4% of carvacrol was retained in the film after 28 days of storage.

The significant release of carvacrol observed in this study from AM starch-based films stored while exposed to the atmosphere may be attributed to its high volatility. According to Han (2005a), the migration rate of volatile AM agents from packaging

film might depend on the chemical interactions between the volatile AM agent and the packaging materials. This behaviour of release to the atmosphere from the AM starch-based films containing carvacrol also provides an indication of the tendency of the AM agent to be released into the packaging headspace (Rardniyom *et al.*, 2008b) where it can reach the surface of the food and perform its AM activity as suggested by Han (2005a).

The storage at ambient conditions of AM films wrapped in aluminium foil demonstrated significant effect on the retention of carvacrol in the MC-HPMC coatings during the storage period. Of course, there is need of AM film to be volatile, however; if it is left in an open environment, there is possibility of AM agent concentration in the film being depleted. This concentration depletion is one of the potential problems facing the commercialisation of this technology at present. One remedy would be to put extra layer and/or high-barrier bags or pouches that can prevent the outward diffusion of the AM agent in the film. However, inside the high-barrier bags or pouches, there would be some loss of AM agent to the free volume in the bag during storage but this could be accounted for. The loss would not be continuous under such conditions as an equilibrium concentration would presumably be reached. Therefore, a barrier layer such as aluminium foil should considerably improve the retention of carvacrol in the MC-HPMC coatings during storage, also for commercial applications. A similar retention of carvacrol was observed by Rardniyom (2008a) who investigated the retention of carvacrol in MC-HPMC coatings. Rupika (2008a) has also reported a significant retention of carvacrol in LDPE films wrapped in aluminium foil. If industry was to

adopt this technology, it would be recommended that a protective barrier layer should be used to prevent excessive loss of AM agent to the atmosphere during storage.

4.11 Antimicrobial Activity Assay on Solid Media

The results for the characterisation of the TPS films demonstrated that these films can be applied to the packaging of foodstuffs that have $a_w < 0.75$. On the other hand, the results obtained for the APTPS film suggest that it can be applied across the entire range of a_w values for packaging of food products. Thus, the APTPS films were coated with AM agents: linalool, carvacrol and thymol and applied to solid media, namely an agar media and Cheddar cheese, each of which has a high a_w value. The AM films were tested *in vitro* in order to provide preliminary information about the potential AM activity of the active agents coated onto the starch-based films against *S. aureus, S. cerevisiae* or *A. niger*. Once it had been confirmed that the AM agents are effective using solid media, the films can then be used to wrap Cheddar cheese inoculated with *S. aureus, S. cerevisiae* or *A. niger*. The preliminary tests were performed using an agar disc diffusion assay (Kuorwel *et al.*, 2011b; Rupika *et al.*, 2005; Suppakul *et al.*, 2008) and the modified microatmosphere method (Guynot *et al.*, 2003).

4.11.1 The AM Activity against *S. aureus* Using Agar Diffusion Assay

An agar disc diffusion assay was used to determine the effectiveness of AM films on the growth of *S. aureus*. The presence of a clear zone of inhibition around the test films was taken as an indication of AM activity in the film formulation. Figure 4.15 shows the AM activity of starch-based films coated with linalool, carvacrol or thymol against *S.*

aureus at 37°C on the solid media in terms of the clear inhibition zones. These zones are visible in the systems containing the AM agent. The film containing no AM agent (control) did not inhibit the growth of *S. aureus* on the solid medium, as expected.

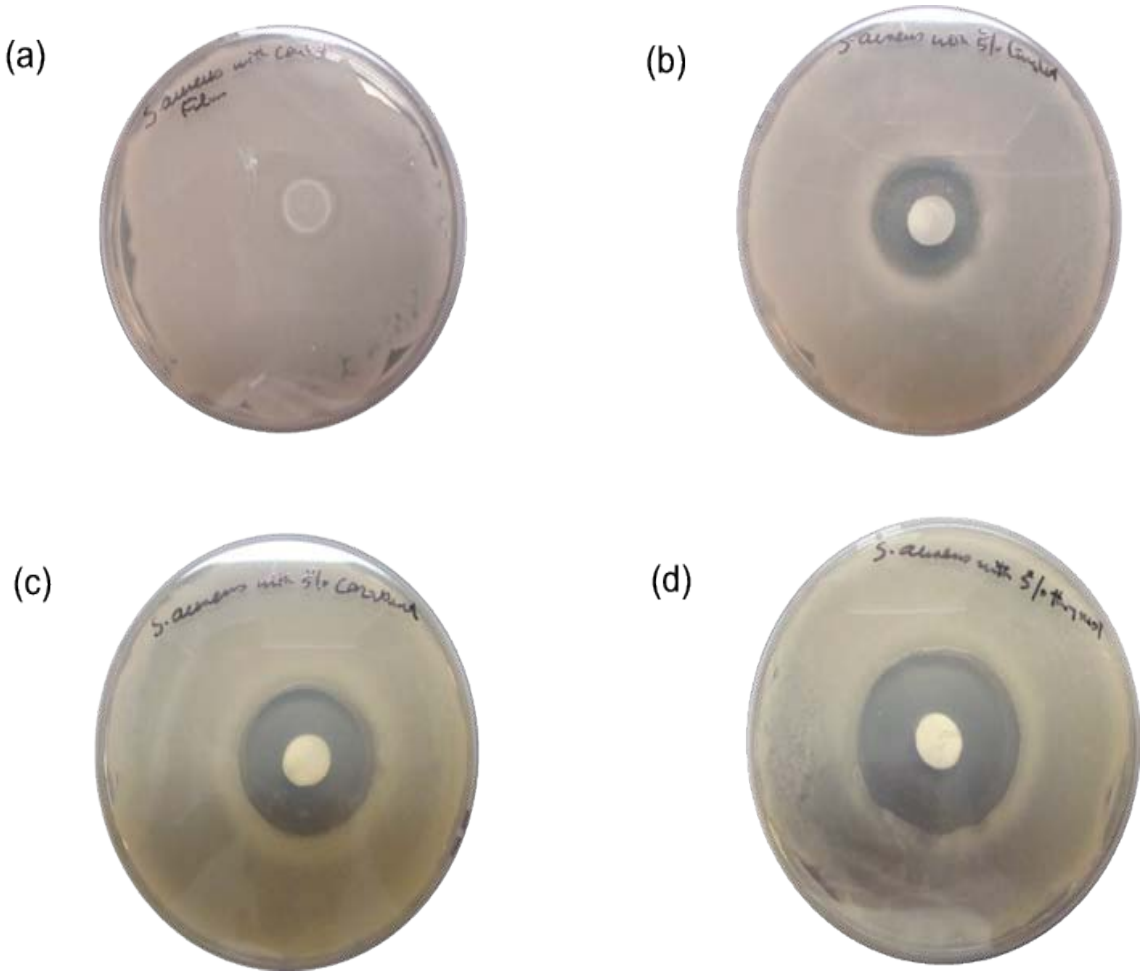

Figure 4.15 Inhibition of *S. aureus* on solid media at 37°C after 24 h on starch-based coated films containing: (a) no AM agent, (b) 2.38% (w/w) linalool, (c) 2.38% (w/w) carvacrol, and (d) 2.38% (w/w) thymol.

The average values of the zones of inhibition for each of the AM films are presented in Table 4.7. It can be seen from Table 4.7 that the inhibitory effect of these agents when coated onto the films increased significantly ($p \leq 0.05$) with the increase in concentration of the agent. All the films coated with carvacrol demonstrated a positive AM activity against *S. aureus* in this study. A detailed statistical analysis of the results suggests that the inhibitory effect of the film containing 0.48% (w/w) carvacrol was significantly ($p \leq 0.05$) lower than that containing 1.43% (w/w) and even more so than the film containing 2.38% (w/w) carvacrol. A similar concentration dependence of carvacrol activity against *S. aureus* on solid media was observed by Rupika *et al*. (2005) who evaluated the AM activity against *S. aureus* of polyethylene films containing carvacrol within the bulk of the film, using the agar disc diffusion assay. In the present study, the inhibitory activity of linalool-coated starch-based film against *S. aureus* increased notably with increasing concentration. The zone of inhibition data for each of the concentrations showed that the films containing 2.38% (w/w) linalool, carvacrol or thymol had a higher inhibitory activity than those containing any of the lower concentrations of these agents in their coatings and were all effective against *S. aureus* on solid media. The greatest inhibition for the starch-based coated films occurred with 2.38% (w/w) thymol. This observation is consistent with the work of Sivropoulou *et al*. (1995). Tepe *et al*. (2004) have also reported a significant AM activity of thymol against *S. aureus* also *in vitro*.

Table 4.7 Analysis of the zone of inhibition data in solid media for *S. aureus* at 37°C in the presence of starch-based film coated with the AM agents: carvacrol, linalool or thymol.

Treatment	Zone of inhibition/mm *S. aureus*			Gradient dz/dc	Intercept	Correlation coefficient (R^2)
	0.48% (w/w)	1.43% (w/w)	2.38% (w/w)			
Linalool	9.2 ± 0.3^a	13.3 ± 0.6^b	18.6 ± 1.0^c	5.98	-4.69	0.969
Carvacrol	10.3 ± 1.1^a	15.3 ± 1.7^b	21.9 ± 1.7^c	7.06	-5.78	0.977
Thymol	11.3 ± 1.8^a	16.7 ± 0.9^b	23.8 ± 1.8^c	7.69	-6.29	0.977

Values for zone of inhibition are represented as Mean ± standard deviation (n=3). Means in the same row for each treatment followed by different letters are significantly different (p = 0.05).

Figure 4.16 shows the variation in the zone of inhibition as a function of concentration of linalool, carvacrol or thymol AM agents. The AM activity of the starch-based films is highly linear between *ca.* 0.48% (w/w) and 2.4% (w/w) as revealed by the linear regression analysis data listed in Table 4.7. These regression data, namely the gradient and the vertical axis intercept, pertain to the zone of inhibition data obtained in the latter concentration range. The respective gradients of the regression lines are indicative of the sensitivity of the test microorganism to changes in the concentration of the three AM agents in the film coating. The order of concentration sensitivity found is: thymol > carvacrol > linalool. Such linearity in the response of *S. aureus* to changes in AM concentration does not, however, seem to be maintained in the region between the

control sample and *ca*. 0.48% (w/w) of AM agent as shown by the dashed line. The latter is consistent with the observations made by Bagamboula *et al*. (2004) who reported a non-linear relationship between the zone of inhibition of *Shigella sp.* with the concentration of thyme and basil EOs with their main constituents: carvacrol, thymol and linalool. However, it is important to note that the non-linearity reported by Bagamboula *et al*. (2004) was observed over the concentration range spanning over several orders of magnitude (0.01 - 10% w/w) and that the data seem reasonably linear in the AM concentration range used in the present work. A possible cause of this phenomenon includes the role of diffusion kinetics in the observed non-linear relation between the zone of inhibition and the concentration of AM agents in the polymer coating. Furthermore, the non-linear relationship may be due to factors that limit the applicability of the method at low concentrations of AM agents (Suppakul *et al.*, 2003b).

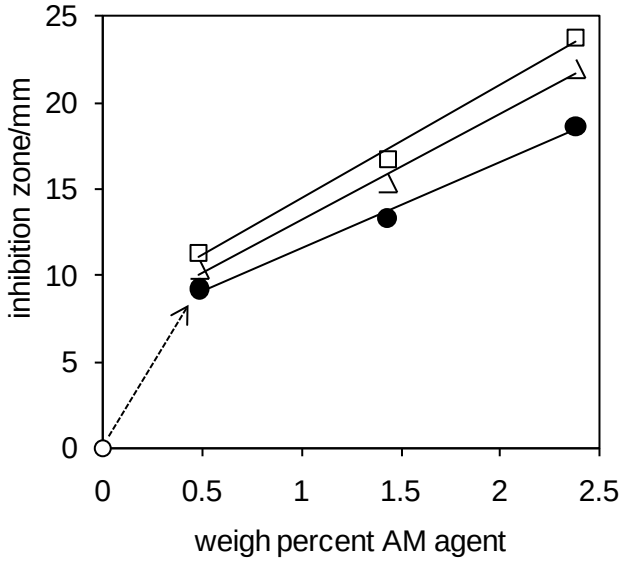

Figure 4.16 Zone of inhibition of *S. aureus* at 37°C versus AM agent concentration for starch-based films containing in their coating: no AM agent (○), linalool (●), carvacrol (△) and thymol (□).

4.11.2 The AM Activity against *S. cerevisiae* Using Agar Diffusion Assay

The AM activity of the starch-based films containing the three different AM agents in their coatings was examined by the agar disc diffusion test. Figure 4.17 shows the AM activity of these films in terms of the clear zones that appear around the samples after 48 h incubation at 25°C. Each clear zone indicates the inhibition caused by the release of the AM agent from the film sample. The numerical average values for the zones of inhibition are presented in Table 4.8. As expected, no inhibitory effect against *S. cerevisiae* growth in the agar disc diffusion test was observed in the case of the control film.

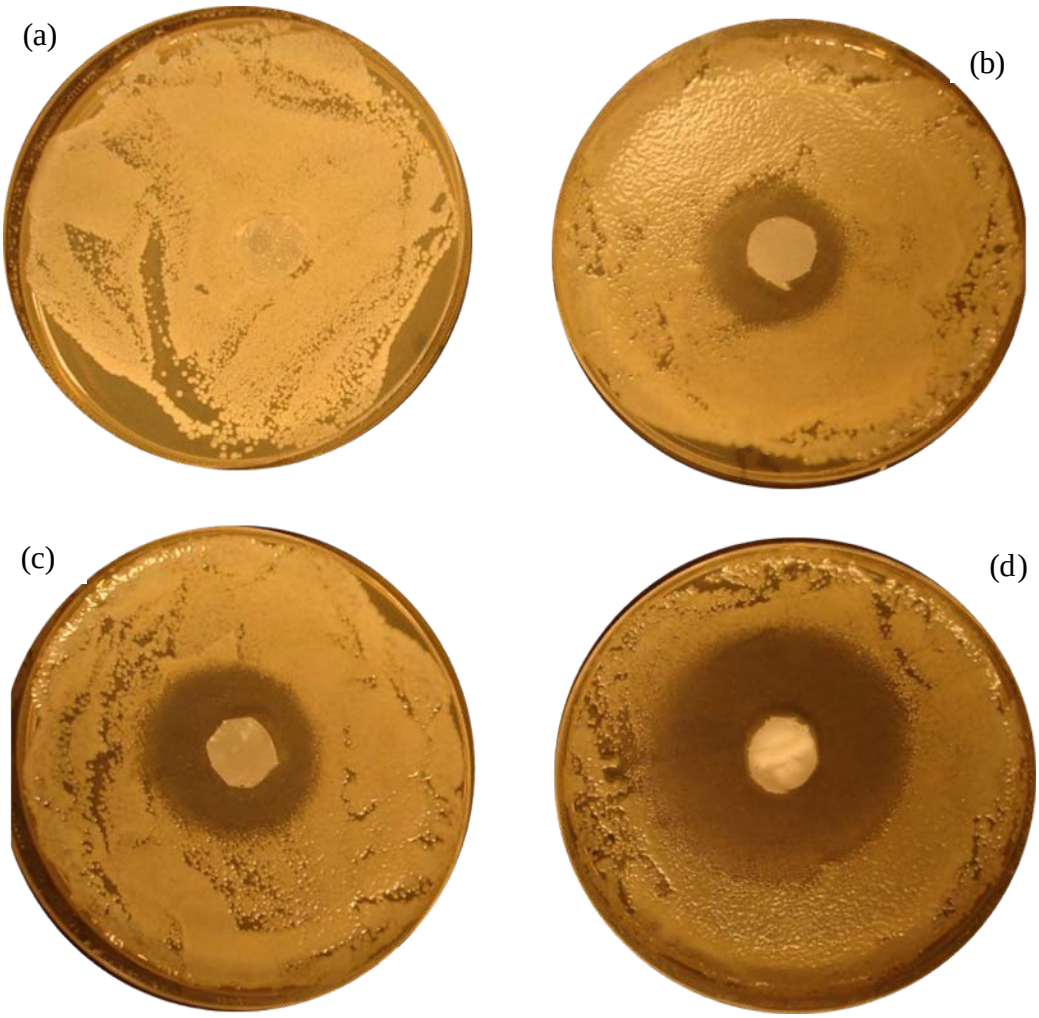

Figure 4.17 Inhibition of *S. cerevisiae* on solid media by starch-based AM films coated with: (a) No AM agent (control), (b) 2.38% (w/w) linalool, (c) 2.38% (w/w) carvacrol, and (d) 2.38% (w/w) thymol, after incubation for 48 h at 25°C.

Table 4.8 Zone of inhibition data in solid media for *S. cerevisiae* at 25°C in the presence of starch-based films coated with the AM agents linalool, carvacrol or thymol at 1, 3 and 5% (w/w) in the coating.

AM agent	Zone of inhibition/mm for *S. cerevisiae*			Gradient dz/dc	Intercept	Correlation coefficient (R^2)
	0.48% (w/w)	1.43% (w/w)	2.38% (w/w)			
Linalool	6.1 ± 0.3^a	8.2 ± 0.9^b	11.2 ± 0.3^c	1.26	4.71	0.988
Carvacrol	7.1 ± 0.4^a	9.3 ± 0.6^b	12.2 ± 0.4^c	1.28	5.69	0.993
Thymol	7.6 ± 0.6^a	10.6 ± 0.9^b	13.2 ± 0.3^c	1.4	6.30	0.999

Values for zone of inhibition are represented as Mean ± standard deviation (n=3). Treatment with different letter within row is statistically significant difference ($p = 0.05$).

The results in Table 4.8 show that the starch-based films coated with thymol were the most effective against *S. cerevisiae* at each of the concentrations tested. The greatest clear zone of inhibition among all samples was achieved in the films coated with 2.38% (w/w) thymol. The AM efficacy demonstrated by thymol here may be due to a number of factors including the ability of thymol to migrate through the media. The starch-based films coated with carvacrol also demonstrated a positive AM activity against *S. cerevisiae* on the solid media. The AM activity of carvacrol against *S. cerevisiae* increased significantly ($p \leq 0.05$) with the increase in concentration in the film coating. These observations are consistent with the study of Rupika *et al.* (2005) who found that

polyethylene-based films containing carvacrol and/or thymol demonstrated significant inhibitory activity against *S. cerevisiae* using the agar disc diffusion assay. The AM activity of starch-based films coated with linalool at 0.48, 1.43 and 2.38% (w/w) were also found to be effective against the growth of *S. cerevisiae* on solid media (see Table 4.8). In these systems the zone of inhibition increased with the increase in linalool concentration from 0.48% to 2.38% (w/w). The data indicate that the inhibitory effect of the films containing linalool against *S. cerevisiae* on the solid medium is significantly less than that of the films containing thymol in all cases.

In order to assess the relative effectiveness against the growth of *S. cerevisiae* of the three natural AM agents, the zone of inhibition data in Table 4.8 were plotted against the concentration of AM agent as shown in Figure 4.18. The effectiveness response of each of these systems to an increase in concentration was found to be linear over the tested concentration range. However, the response was non-linear in the concentration range between zero and 0.48% (w/w) of AM agents as demonstrated by the non-zero axial intercept of each plot. The gradients of these plots reflect the sensitivity of *S. cerevisiae* to changes in the concentration of the AM agents. The latter results suggest that this microorganism is equally sensitive to concentration changes in the linalool and carvacrol systems with a slightly higher sensitivity (*ca*. 10%) in the thymol system. A statistical analysis of the results showed that at the 0.48% (w/w) level of AM agents, there were significant differences in the effectiveness between linalool and carvacrol or thymol but no significant differences between the carvacrol and thymol systems. In contrast, significant differences were found amongst all three systems at the 2.38% (w/w) level of AM agent.

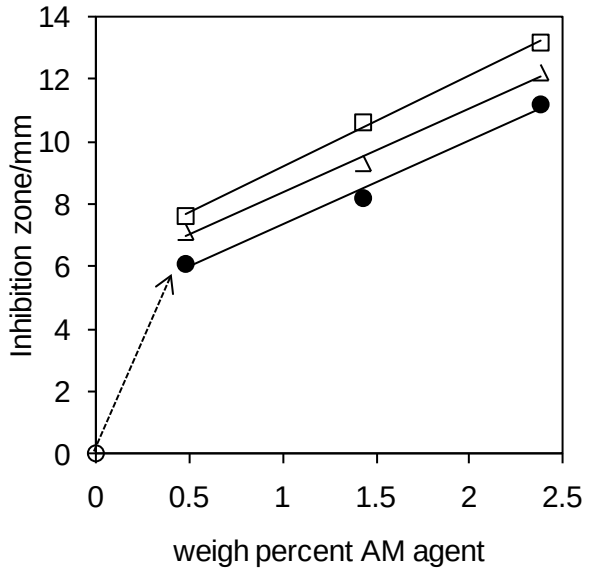

Figure 4.18 Plot of the zone of inhibition of *S. cerevisiae* versus concentration of AM agents for starch-based films containing: no AM agent (○), linalool (●), carvacrol (△) and thymol (□) in their coating. The zones of inhibition were created on solid media after incubation for 48 h at 25°C.

4.11.3 The Antifungal Activity Using Modified Microatmosphere

The AM films were initially tested on a solid medium before being used in the experiments involving Cheddar cheese. The antifungal activity of linalool, carvacrol or thymol in the coatings of the starch-based film at three different levels 0.48%, 1.43% and 2.38% (w/w) was tested against *A. niger* using the modified microatmosphere method. All three systems demonstrated antifungal activity against *A. niger* and the inhibitory effect of the systems showed a wide variation amongst the AM agents. The degree of inhibition, as indicated by the size of the colony diameter, increased with the increase in concentration of AM agent. Figure 4.19 shows photographs depicting the antifungal activity of the starch-based films. The film containing no AM agent in the coating exhibited no appreciable inhibition of the *A. niger* as indicated by the large

fungal growth on the surface of the solid medium (see Figure 4.19(d)). The mean colony diameter for this system and the colony diameters of the other AM systems are presented in Table 4.9 for comparison.

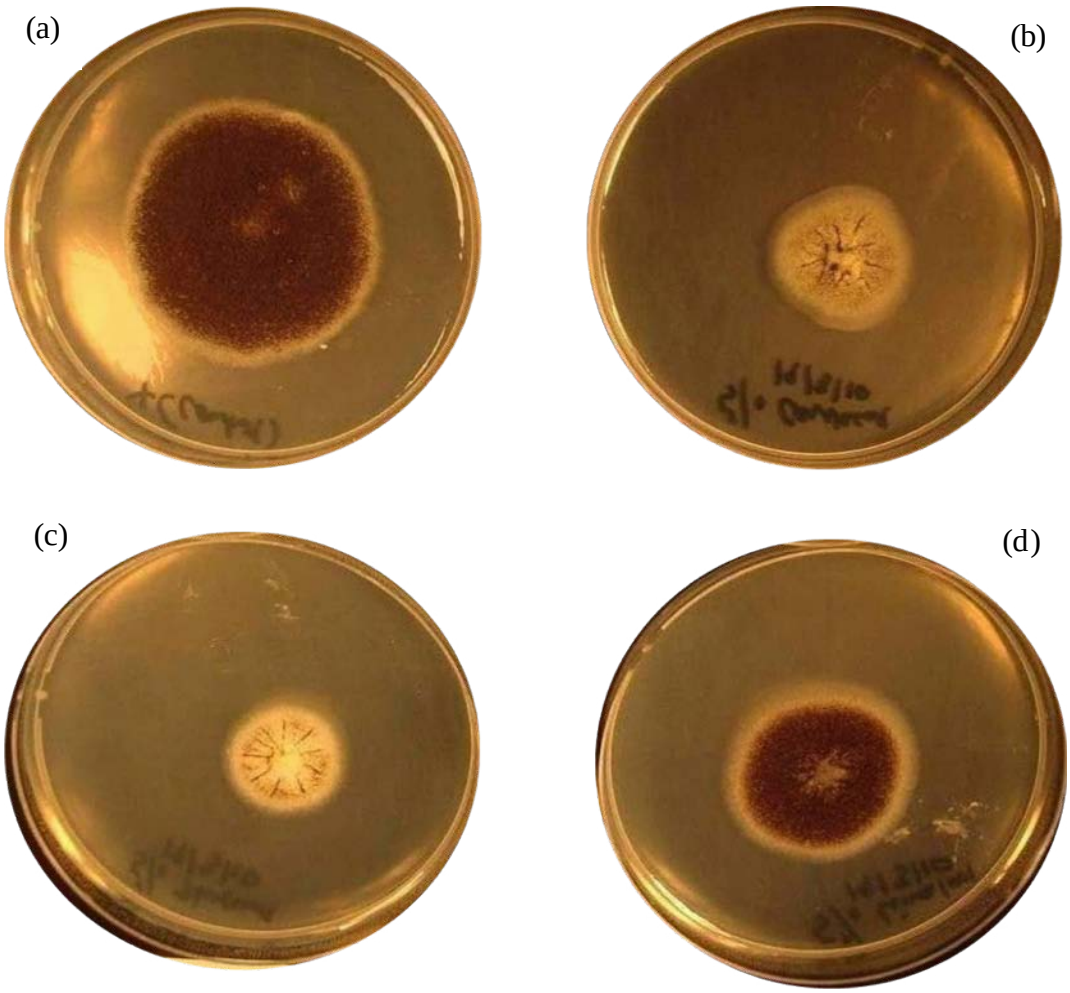

Figure 4.19 Inhibition of *A. niger* on solid media at 25°C after 7 d by starch-based AM films containing: (a) The control film (contained no AM agent in its coating), (b) 2.38% (w/w) carvacrol, (c) 2.38% (w/w) thymol, and (d) 2.38% (w/w) linalool, in their coating.

Table 4.9 Colony diameter data for *A. niger* (5.75×10^6 spores mL^{-1}) on solid media at 25°C in the presence of starch-based film containing the AM agents linalool, carvacrol or thymol in their coatings.

	Colony Diameter/mm			
AM Agent	Concentration of AM Agent in coating			
	0% (w/w)	0.48% (w/w)	1.48% (w/w)	2.38% (w/w)
Control	85.3 ± 2.1	-	-	-
Linalool		49.2 ± 0.6^a	39.7 ± 0.9^b	29.3 ± 0.9^c
Carvacrol		45.2 ± 0.6^a	36.3 ± 0.4^b	25.4 ± 1.8^c
Thymol		42.4 ± 1.5^a	33.7 ± 1.2^b	21.3 ± 1.0^c

Values for colony diameter are represented as mean ± standard deviation (n=3). Treatment with same letter within row is not statistically significant difference ($p = 0.05$)

The data presented in Table 4.9 demonstrate that the inhibitory activity of starch-based films containing linalool or carvacrol in their coatings against *A. niger* increased significantly with increasing concentration. The inhibitory effect of thymol contained in the film coatings increased significantly ($p \leq 0.05$) with the increase in the AM agent concentration. This result is consistent with the concentration dependence of thymol activity against *A. niger* on solid media observed by Rupika *et al.* (2005) for the AM activity of polyethylene films containing thymol against *A. niger* using the agar disc diffusion assay. The relative colony diameter values indicate that the starch-based films containing thymol in their coatings were most effective toward *A. niger* followed by carvacrol and then linalool. The observed inhibition of *A. niger* by carvacrol is

consistent with the work of López-Malo *et al.* (2005) on the antifungal activity of carvacrol against the growth of *A. Flavus* on potato dextrose agar. Our observations are also consistent with the results of Bagamboula *et al.* (2004) on the inhibitory effect of thymol against *Shigella sp.* on solid media. A high antifungal sensitivity of thymol has also been reported by López-Malo *et al.* (2002).

The antifungal activity of these systems was also investigated in terms of a linear regression analysis of the colony diameter data. The relative average colony diameters given in Table 4.9 were plotted as a function of concentration to determine the sensitivity of *A. niger* to changes in the concentration of AM agent in the film coatings. These plots are shown in Figure 4.20 from which a significant dose dependency for each of the AM agents across the studied concentration range is evident.

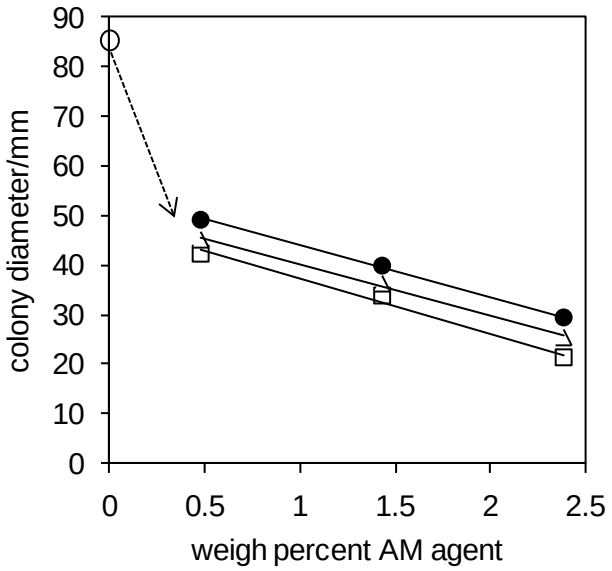

Figure 4.20 Plot of the colony diameters versus concentration of AM agents for starch-based films containing: no AM agent (○), linalool (●), carvacrol (△) and thymol (□) in their coating. The colony diameters were observed on solid media after incubation for 7 d at 25°C.

The plots in Figure 4.20 indicate that the effectiveness of each system is non-linear between zero and 0.48% (w/w) concentration of the AM agent in the coatings. However, the marked decrease in the colony diameter that occurs over this small concentration range reflects the high fungicidal efficacy of the AM agents in the systems. In the concentration range of 0.48% (w/w) to 2.38 % (w/w) the concentration dependence is linear in all cases. The respective gradients of these plots reflect the sensitivity of *A. niger* to changes in the concentration of the AM agent in the respective coating. The sensitivities of all three systems are similar with those of linalool and carvacrol being almost identical and that of thymol being slightly higher. A detailed analysis showed that there is a statistical significant difference between the effectiveness of thymol compared with linalool or carvacrol over the tested concentration range. Given: (i) the superior effectiveness of thymol as a fungicidal agent against *A. niger* as reflected by the colony diameter data and (ii) the slightly greater sensitivity of *A. niger* to changes in the thymol concentration compared with the other two AM agents, it is suggested that the thymol system exhibits an overall superiority as a fungicide against *A. niger* on a solid medium substrate. The strong inhibitory effect demonstrated by thymol in the coating of the starch-based film may be attributable to its ability to diffuse uniformly through an agar media as suggested by Bagamboula *et al.* (2004). However, Friedman *et al.* (2002) pointed out that volatile compounds such as thymol exhibit poor solubility in the aqueous phase.

4.12 Antimicrobial Activity on Cheddar Cheese - A Challenge Test

The production of the AM films using the coating technique resulted in films that exhibit positive AM activity on solid media. Since all the AM films showed positive inhibitory effects against *S. aureus, S. cerevisiae or A. niger* on solid media, the effect of the AM films on a real food was explored. The AM films were used to package Cheddar cheese in order to assess their effectiveness against the respective microorganisms on the cheese samples and the results are presented in this section.

4.12.1 The Antimicrobial Activity against *S. aureus* on Cheddar Cheese

Further to the *in vitro* study using the agar diffusion technique, the effect of the AM starch-based films was explored when placed in contact with a particular foodstuff. In particular, the films were used to package samples of Cheddar cheese in order to assess their effectiveness against *S. aureus* that was inoculated on the surface of the samples and in order to attempt to identify low concentrations where effective growth control of the microorganisms occurs. Clearly, a low concentration of additive is preferable since the higher the concentration of the natural plant extracts the greater is the concern about off-flavour issues for food packaging applications (Suppakul *et al.*, 2003a). Figures 4.21 (a) is a plot of the decadic logarithm of the population counts of *S. aureus* on the surface of the cheese as a function of storage time (i.e. the "death" curves) at 15°C for starch-based AM films containing coatings of linalool. Data for the control film are also plotted for comparison. Similar trends were observed for the death curves obtained using carvacrol and thymol-coated starch-based films, as shown by Figures 4.21(b) and (c) respectively. The specific death rates were determined from the gradients of the

plots for all the systems. These data are listed in Table 4.10 along with the other linear regression analysis data.

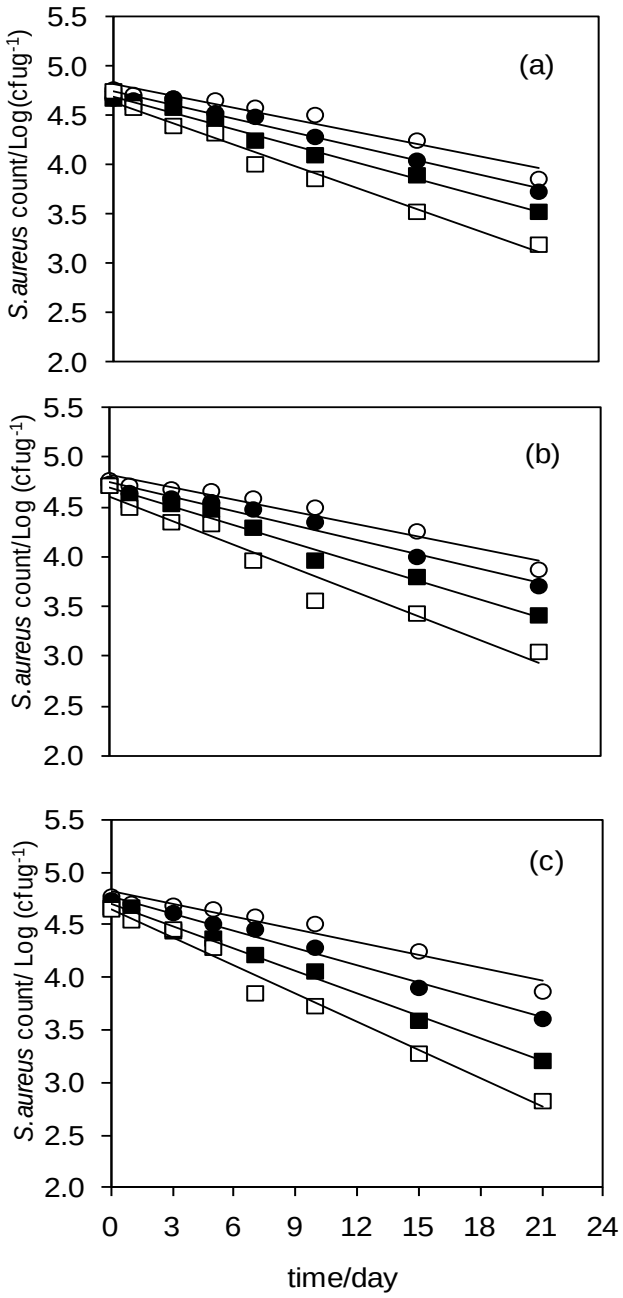

Figure 4.21 Inhibition of *S. aureus* on Cheddar cheese packaged and stored at 15°C in AM starch-based films coated with AM agents: (a) linalool, (b) carvacrol and (c) thymol; each containing: a control film (○), (●) 0.48% (w/w), (■) 1.43% (w/w) and (□) 2.38% (w/w) in their coatings.

Table 4.10 Analysis of the "death" curve data for *S. aureus* on the surface of Cheddar cheese packaged and stored at 15°C in starch-based films coated with AM agents: linalool, carvacrol or thymol.

Treatment	AM agent concentration in coated film % (w/w)	Specific death rate μ'/day^{-1}	Intercept	Correlation coefficient R^2	Population of *S. aureus* on cheese log CFU g^{-1}	
					Day 0	**Day 21**
Control	0	0.041	4.82	0.951	4.76 ± 0.08	4.06 ± 0.48[a]
Linalool	0.48	0.047	4.74	0.976	4.66 ± 0.10	3.66 ± 0.26[b]
	1.43	0.056	4.70	0.989	4.66 ± 0.10	3.47 ± 0.25[bc]
	2.38	0.072	4.64	0.979	4.74 ± 0.07	3.17 ± 0.13[cd]
Carvacrol	0.48	0.048	4.75	0.977	4.73 ± 0.09	3.7 ± 0.15[b]
	1.43	0.062	4.70	0.980	4.71 ± 0.02	3.36 ± 0.23[bc]
	2.38	0.079	4.60	0.952	4.71 ± 0.08	2.9 ± 0.44[cd]
Thymol	0.48	0.054	4.76	0.984	4.73 ± 0.05	3.6 ± 0.11[b]
	1.43	0.071	4.70	0.994	4.67 ± 0.05	3.12 ± 0.40[bc]
	2.38	0.089	4.64	0.984	4.65 ± 0.04	2.65 ± 0.50[cd]

Means in the same column for each treatment followed by different letters are significantly different (p = 0.05).

The data shown in Table 4.10 confirm the consistency in the initial inoculation procedure as reflected by the consistency in the vertical axis intercepts of the plots. A more detailed analysis of the specific death rates data can be obtained by plotting these values as a function of the concentration of the AM agent for each of the experimental systems. Such a plot is shown in Figure 4.22 where a linear relationship exists between the specific death rate and the concentration of each of the AM agents across the entire range of studied concentrations. This, contrasts slightly to the behaviour exhibited in the solid media where a non-linear relationship was observed between the zone of inhibition and the AM concentration at low concentrations. These observations suggest that the specific death rate μ' may in fact be a pseudo-first order rate constant where $\mu' = \alpha[AM]$, where [AM] is the concentration of AM agent and α is a second-order rate constant. The second order rate constants can be obtained from the gradient of the plots in Figure 4.22.

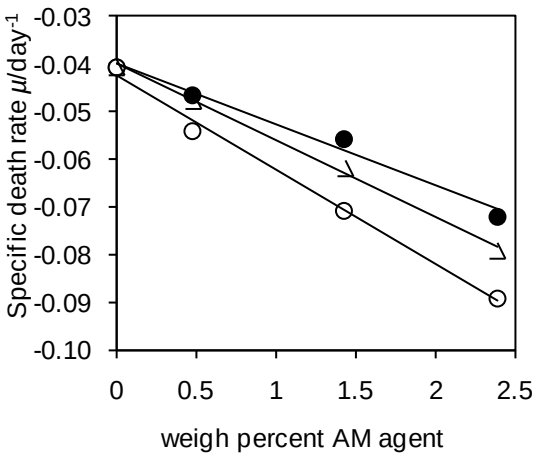

Figure 4.22 Specific death rate of *S. aureus* on the surface of Cheddar cheese versus AM agent concentration for cheese packaged and stored at 15°C in starch-based films containing in their coating: linalool (●), carvacrol (△) and thymol (○).

The plots shown in Figure 4.22 indicate that the inhibition of *S. aureus* on the surface of Cheddar cheese when packed in starch-based films coated with these AM agents is *ca*. 1.6 times more sensitive to changes in the concentration of thymol compared to that of linalool. The sensitivity of this microorganism to changes in carvacrol concentration is between those of the other two AM agents. The relative order of the sensitivity determined in these storage experiments at 15°C was similar to that found in the solid media experiments conducted at 37°C suggesting that the relative order remains unchanged across this range of temperatures.

In the present study the starch-based films containing thymol demonstrated the strongest inhibitory effect on the growth of *S. aureus* on the cheese when compared to the control film (see Table 4.10). The seemingly natural rate of decrease of the *S. aureus* count observed in the control film might be due to the depletion of oxygen during the test and/or other factors such as the depletion of preservatives originally present in the Cheddar cheese. During the first 5 days, the AM films containing 0.48% (w/w) thymol in their coatings extended the lag phase of *S. aureus* growth and reduced by 24% the *S. aureus* population on the cheese after 21 days of storage. The AM films containing thymol at 1.43% (w/w) and 2.38% (w/w) in their coatings further extended the lag phase and reduced the *S. aureus* count on the surface of the Cheddar cheese by 33% and 43% respectively. A high AM activity of thymol has also been reported by Olasupo *et al*. (2003) and is consistent with the present findings.

The population count of *S. aureus* on the cheese packaged in the starch-based film containing 0.48% (w/w) linalool decreased by 22% after 21 days of storage at 15°C (see

Table 4.10). It can also be seen from the results in Table 4.10 that increasing the concentration of linalool contained in the film to 1.43% or 2.38% (w/w) had a significant effect; the population of *S. aureus* on the cheese was reduced by 26% and 33% respectively after 21 days of storage. Kim and *et al*. (1995b) observed a similar dose-related activity of linalool against *S. aureus*. Mazzanti *et al*. (1998) as well as by Dorman and Deans (2000) have also reported the overall effectiveness of this agent against *S. aureus*. The observations in the present study are also consistent with the results of Rupika *et al*. (2006) who found that linalool exhibited an inhibitory effect against *S. aureus* on Cheddar cheese packaged in polyethylene-based AM films. The observed inhibition of *S. aureus* on the surface of Cheddar cheese by carvacrol is consistent with the work of Rardniyom (2008a) who reported the AM activity of carvacrol against the growth of *E. coli* on Cheddar cheese. The linear response in the inhibition of *S. aureus* with the concentration of carvacrol (see Figure 4.22) is in accordance with the observations made by Ultee *et al*. (1998) who found a concentration dependence of carvacrol against *B. cereus* as well as those of Periago *et al*. (2001) who observed a dose-dependence of carvacrol activity also against *L. monocytogenes* in carrot juice.

4.12.2 Antimicrobial Activity against *S. cerevisiae* on Cheddar Cheese

Given that APTPS films coated with linalool, carvacrol and thymol demonstrate an inhibitory effect against *S. cerevisiae* on solid media using the agar diffusion assay, their inhibitory effect on Cheddar cheese was then explored in order to determine their potential for preserving food products from contamination caused by yeasts. The coated starch-based films were evaluated for their AM activity on Cheddar cheese inoculated

with *S. cerevisiae*. The challenge test on the cheese was performed at 15°C in order to determine the activity of the three AM agents against *S. cerevisiae* under temperature abuse conditions. The AM efficacy of the films was compared quantitatively in accordance with the method described by Bachrouri *et al.* (2002). Figure 4.23 (a) is a plot of the decadic logarithm of the population counts of *S. cerevisiae* on the surface of the cheese as a function of storage time in the starch-based AM films incorporating linalool in their coatings. Similar curves were obtained for the death curves of the films that incorporated carvacrol and thymol in their coatings (see Figure 4.23(b) and (c)) respectively. Data for the control film are also plotted for comparison.

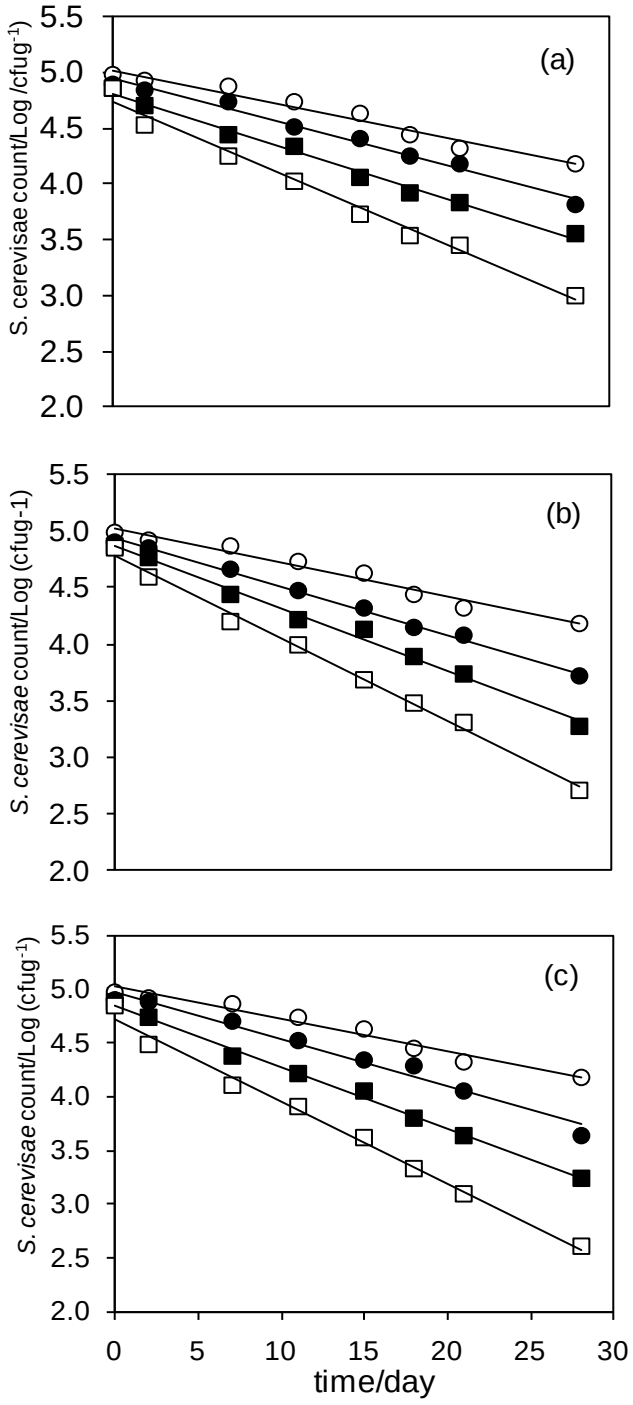

Figure 4.23 Inhibition of *S. cerevisiae* on the surface of Cheddar cheese packaged and stored under temperature abuse conditions of 15°C in starch-based films with a coating containing AM agents: (a) linalool, (b) carvacrol and (c) thymol at (●) 0.48% (w/w), (■) 1.43% (w/w) and (□) 2.38% (w/w). The control film (○) containing no AM agent is shown for the comparison.

These results suggest that all the starch-based films containing linalool in their coating had a significant ($p \leq 0.05$) inhibitory effect on the growth of *S. cerevisiae* on Cheddar cheese at all tested concentrations compared to the control film. The results also suggest a decline of the *S. cerevisiae* population count on the cheese during the 28 days of storage at 15°C. The starch-based film containing linalool in its coating reduced the *S. cerevisiae* population count on the cheese samples by 1.19, 1.41 and 1.93 log(CFU g^{-1}) units for the films containing 0.48%, 1.43% and 2.38% (w/w) of linalool respectively during the 28 days of storage at 15°C. In comparison, the population count of *S. cerevisiae* for the cheese samples wrapped in the control film decreased by 0.8 log(CFU g^{-1}) units only when stored under identical conditions.

Further to the AM activity of linalool, the population of *S. cerevisiae* on the Cheddar cheese samples was found to be significantly ($p \leq 0.05$) lower when packed in starch-based films containing carvacrol in their coating at each of the three concentration levels tested. The population of the *S. cerevisiae* samples wrapped with films containing 0.48, 1.43 and 2.38% (w/w) carvacrol in their coating decreased by 24.2%, 32.9% and 43.8% respectively after 28 days of storage at 15°C compared with the control film where the population count decreased by 11% only over the same period. This corresponds to a reduction of 1.2, 1.6 and 2.4 log(CFU g^{-1}) units for the systems containing 0.48, 1.43 and 2.38% (w/w) of carvacrol in their coatings compared with the control film (i.e. 0.8 log(CFU g^{-1})). Moreover, the starch-based films containing thymol at 0.48, 1.43 and 2.38% (w/w) in their coatings also significantly reduced the population of *S. cerevisiae* inoculated on the cheese by 1.4, 1.8 and 2.7 log(CFU g^{-1}) units respectively compared to the control film (i.e. 0.8 log(CFU g^{-1})). In accordance with the

results in the agar diffusion assay, thymol demonstrated the highest AM inhibitory effect, followed by carvacrol and then linalool that demonstrated the least inhibitory effect against *S. cerevisiae* on Cheddar cheese. These observations are also in agreement with the results of Kuorwel *et al.* (2010) who reported the AM activity of starch-based films containing linalool, carvacrol or thymol in the film coatings against *S. aureus* to be dependent on the concentration of the AM agent in the coating.

Table 4.11 lists the linear regression parameters from the analysis of the specific death rate data obtained for *S. cerevisiae* on the surface of Cheddar cheese that was wrapped in starch-based films containing 0.48, 1.43 or 2.38% (w/w) linalool, carvacrol or thymol in their coatings and stored under temperature abuse conditions of 15°C for up to 28 days. The data show a high degree of reproducibility within the experiments as indicated by the consistent values of the vertical axis intercepts. Specifically, the consistency of these data confirms the consistency in the initial inoculation procedure.

Table 4.11 Linear regression parameters from the analysis of death rate data for *S. cerevisiae* on the surface of Cheddar cheese wrapped in starch-based films containing 1, 3 or 5% (w/w) linalool, carvacrol or thymol in their coatings and stored under temperature abuse conditions of 15°C for up to 28 days.

Treatment	Formulation % (w/w)	Specific death rate μ/day^{-1}	Intercept	Correlation coefficient (R^2)
Control	0	0.030	5.02	0.971
Linalool	0.48	0.038	4.94	0.986
	1.43	0.047	4.81	0.991
	2.38	0.063	4.73	0.987
Carvacrol	0.48	0.043	4.94	0.996
	1.43	0.055	4.87	0.991
	2.38	0.073	4.78	0.996
Thymol	0.48	0.044	4.97	0.978
	1.43	0.058	4.85	0.996
	2.38	0.077	4.73	0.992

In order to explore in greater detail the effect of changes in the concentration of AM agent in the film coating on the death rate in these systems, the specific death rate data given in Table 4.11 were plotted as a function of the AM concentration in the film coating as shown in Figure 4.24.

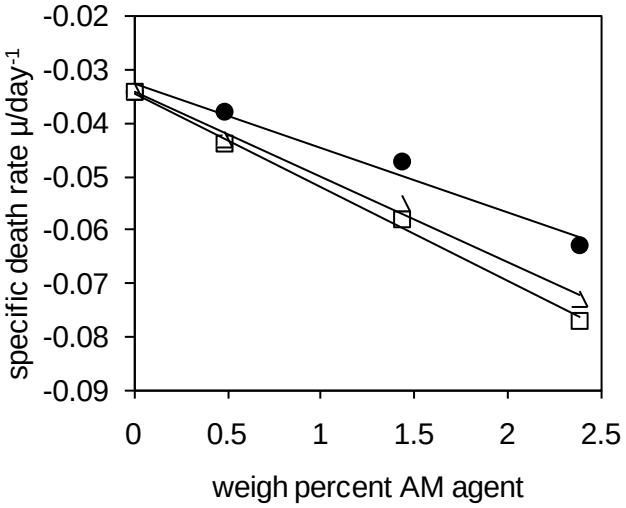

Figure 4.24 Plot of the death rate of *S. cerevisiae* on the surface of Cheddar cheese as a function of the concentration of: (●) linalool, (Δ) carvacrol and (□) thymol present in the surface coatings of starch-based films that were used to wrap the cheese samples under temperature abuse conditions of 15°C.

Figure 4.24 shows that the sensitivity of *S. cerevisiae* to changes in the concentration of AM agent in the film coatings remained constant over the concentration range investigated in this study. The figure also reveals a *ca*. 7% higher sensitivity of this organism to changes in the concentration of thymol compared with linalool. A statistical analysis of the results suggests that although a significant difference exists between the sensitivity of *S. cerevisiae* to thymol and linalool, no such significant difference exists between its sensitivity to carvacrol and linalool. Furthermore, the data suggest the overall relative order of the sensitivities determined in these storage experiments at 15°C is the same as that found in the case of the solid media experiments conducted at 25°C suggesting that the relative order remains unaffected within this temperature range.

4.12.3 The Antifungal Activity of AM agents on Cheddar Cheese

One of the aims of the present study was to determine whether the growth of *A. niger* on Cheddar cheese could be inhibited by APTPS films containing various concentrations of linalool, carvacrol and thymol in their coatings. If the Cheddar cheese samples wrapped in the AM films exhibited a reduced growth rate of *A. niger* compared with the control film, it can therefore be suggested that the APTPS films containing AM agents in their coatings are effective in maintaining the quality and safety of cheese and/or other dairy products. To this end, the antifungal activity of the coated starch-based AM films against *A. niger* inoculated on the surface of Cheddar cheese samples stored at the temperature abuse conditions of 15°C was explored. Figure 4.25 (a) shows plots of the decadic logarithm of the *A. niger* count on the surface of Cheddar cheese samples as a function of the time of storage where the samples were packaged and stored at 15°C in starch-based AM films containing linalool in their coatings at three different concentrations.

Data for samples packaged and stored in the control film containing no linalool in its coating are also shown for comparison. Similar trends were observed for samples stored in films containing carvacrol or thymol in their coatings (see Figure 4.25(b) and (c)) respectively. The plots show a high consistency in the initial inoculation technique of the samples as revealed by the consistent vertical axis intercepts.

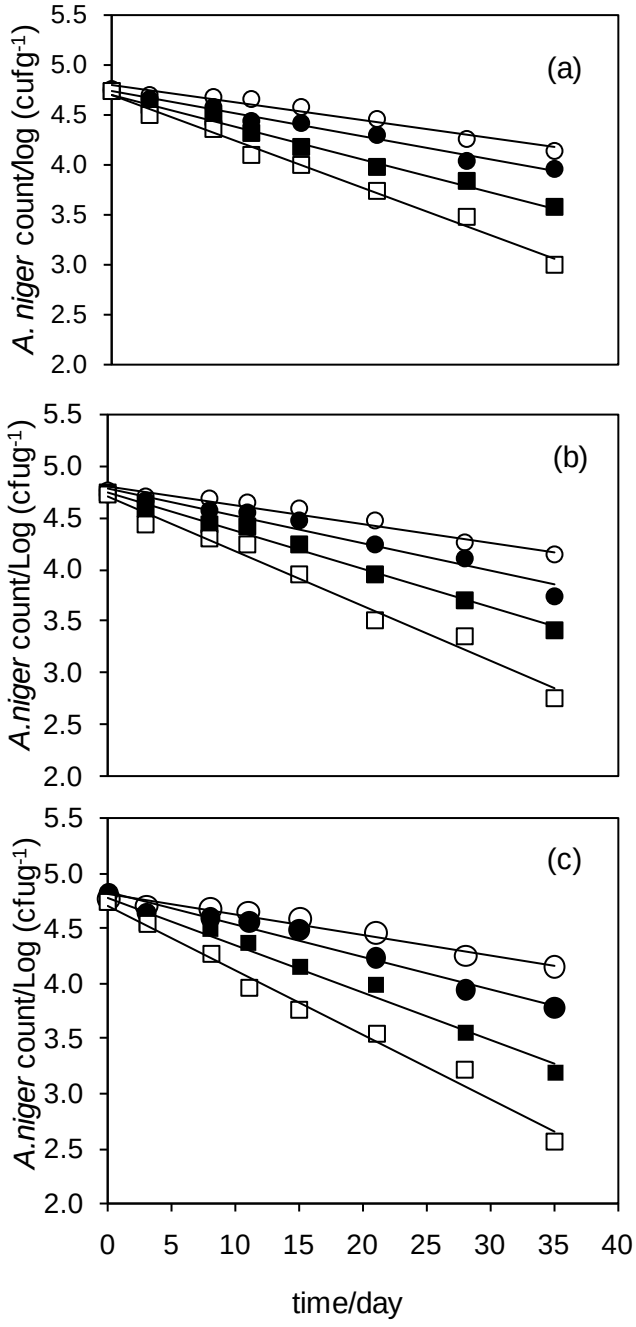

Figure 4.25 Inhibition of *A. niger* on the surface of Cheddar cheese packaged and stored at 15°C in starch-based films containing AM agents: (a) linalool, (b) carvacrol and (c) thymol at the concentration of (●) 0.48% (w/w), (■) 1.43% (w/w) and (□) 2.38% (w/w) in their coatings. Data for the control film (containing no AM agent in its coating are also plotted.

Each of the starch-based films containing linalool inhibited significantly ($p \leq 0.05$) the growth of *A. niger* on the surface of Cheddar cheese and the inhibitory effect was observed to be a function of concentration of the AM agent (Figure 4.25). A detailed analysis showed that the starch-based films containing 0.48%, 1.43% or 2.38% (w/w) linalool in their coatings inhibited the growth of *A. niger* by 17%, 24% and 37% respectively, after 35 days of storage at 15°C compared with the control film where the population count of *A. niger* on the cheese decreased by 12% over the same period. The addition of carvacrol at 0.48%, 1.43% or 2.4% (w/w) into the film coating resulted in the reduction in the *A. niger* count on the cheese surface by 21%, 28% and 41% respectively after 35 days at 15°C. As expected, the population of *A. niger* on Cheddar cheese samples reduced significantly ($p \leq 0.05$) further when samples were wrapped in the starch-based films containing thymol in their coating at each of the three concentration levels tested. In this case, the population count of *A. niger* decreased by 22%, 32% and 46% in the cheese packaged in films containing 0.48%, 1.43% or 2.38% (w/w) respectively of thymol in their coating and at the same conditions.

To explore in greater detail the inhibitory effect of each AM agent coated onto the starch-based films with respect to changes in the concentration, the death rates for *A. niger* on the surface of Cheddar cheese were determined from the linear regression analysis of the data appearing in Figure 4.25. These results are presented in Table 4.12. To further compare the effectiveness of each AM agent against *A. niger* on the surface of Cheddar cheese, the specific death rates presented in Table 4.12 were plotted as a function of the AM concentration in the film coating as shown in Figure 4.26.

Table 4.12 Linear regression analysis of the "death" curve data for *A. niger* inoculated on the surface of Cheddar cheese packaged and stored at 15°C in starch-based films containing the AM agents linalool, carvacrol or thymol in their coatings.

System	AM Agent in Film Coating % (w/w)	Specific Death Rate, μ/day^{-1}	Vertical Axis Intercept	Correlation Coefficient, R^2
Control	0	0.018	4.807	0.964
Linalool	0.48	0.023	4.734	0.984
	1.43	0.032	4.696	0.988
	2.38	0.047	4.701	0.988
Carvacrol	0.48	0.027	4.784	0.952
	1.43	0.037	4.748	0.990
	2.38	0.053	4.710	0.981
Thymol	0.48	0.029	4.827	0.971
	1.43	0.043	4.780	0.981
	2.38	0.059	4.710	0.985

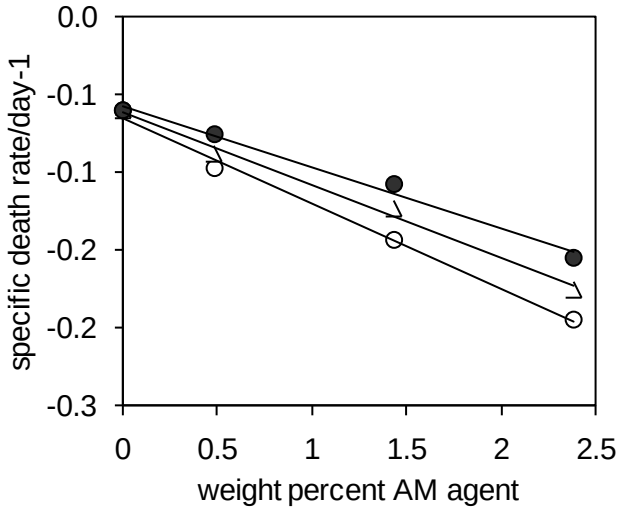

Figure 4.26 Plot of the death rate of A. niger on the surface of Cheddar cheese as a function of concentration of the AM agent in the film coating. The cheese was packaged and stored at 15°C in starch-based films containing the AM agent(■) (linalool, (□) carvacrol and (●) thymol in their coatings.

Figure 4.26 is plot of the death rate of *A. niger* on the surface of the cheese packaged in films coated with linalool and stored at 15°C as a function of the AM agent concentration. This plot also contains a more detailed analysis of the death rate of *A. niger* determined on the surface of the cheese packaged in films coated with carvacrol and thymol. In contrast to the results obtained using the solid media, the inhibitory effect on the surface of the cheese sample of each of the AM agents present in the film coatings is linear across the entire concentration range studied, namely between zero % (w/w) and 2.38% (w/w) (see Figure 4.26). The data confirm the order of effectiveness of the systems in terms of the relative sensitivities of *A. niger* to changes in the

concentration of AM agent in each of the coatings. The highest sensitivity occurred in the thymol and the lowest sensitivity in the linalool system as was observed in the solid media experiments. A detailed analysis of the data plotted in Figure 4.26 also shows that the inhibition of *A. niger* on the surface of Cheddar cheese when wrapped in starch-based films coated with thymol was *ca*. 17% and 8% more sensitive to changes in the concentration than that of linalool and carvacrol respectively. This result is consistent with the results obtained in the solid media experiments. The relative order of sensitivity observed in this study is also consistent with the results of Kuorwel *et al.* (2011b) who studied the AM activity of starch-based films containing linalool, carvacrol and/or thymol in their coatings against *S. cerevisiae* on Cheddar cheese.

5 Conclusion

5.1 General Conclusions

The findings of this study underscore the potential of incorporating natural AM agents (linalool, carvacrol or thymol) into starch-based films. In term of commercialisation, heat sealability is an issue in biobased films; however, The APTPS film coated in this study is heat sealable. Overall, the results indicate that the AM agents linalool, carvacrol or thymol can be successfully coated onto commercially available APTPS films to produce AM packaging films that exhibit activity against *S. aureus*, *S. cerevisiae* and *A. niger in vitro* and on the surface of cheddar cheese. The following are conclusions drawn for the specific objectives of this study.

Studies on the effects of water uptake by TPS films and APTPS films showed that the TPS film has a high affinity for water and tends to dissolve in mixtures of high water content with a consequent deterioration in its mechanical properties. In assessing the effects of RH, the conditioning of TPS film at various RH levels produced a noticeable absorption of moisture at high RH levels. In contrast, the APTPS film did not demonstrate discernible decrease in its mechanical properties when immersed in any of the water-based systems or when conditioned at any of the RH values used in the present study. The mechanical properties of the APTPS film, especially its modulus of elasticity, were found to be significantly higher than those of the TPS film. Due to moisture sensitivity, films derived from TPS material might be suited for packaging of food products of low a_w. Given the inherent stability in all conditions tested, the

APTPS films have the potential to be used in a wide range of food packaging applications.

Similarly to the effects of water content or RH, the tensile strength and Young's modulus of the TPS films was found to decrease and the elongation at break found to increase with an increase in the concentration of AM agents in the formulations. The reduction in the tensile strength of the films might be due to the interactions between the AM agents and the TPS matrix affecting the intermolecular forces within the matrix. The effect of linalool, carvacrol and thymol on the WVP of the films was found to increase with the increase in the concentration of the AM agent in the film. The increase of WVP due to the incorporation of AM agents may be attributed to the structural modifications of the starch network occurring when AM agents (linalool, carvacrol and thymol) are added. The melting temperatures of the TPS films were reduced by the incorporation of linalool, carvacrol and thymol at the concentrations of 2.9%, 3.5% and 3.8% (w/w) respectively. These concentration levels might decrease the intermolecular interaction within the film caused an observed decrease in the degree of crystallinity. The incorporation of the AM agents at any of the three different concentrations did not have a significant effect on the film transparency. However, the incorporation of AM agents at levels >6% (w/w) during film formulation may adversely affect the transparency of the TPS film.

From the quantification of AM agents in the experiments on films, there was a significant loss of AM agents incorporated into the TPS films when prepared by heat pressing which might be due to high volatility during processing. The average retention

of linalool, carvacrol and thymol in the TPS film after heat pressing was *ca.* 36%, 43% and 45% respectively. In the MC-HMPC films, there was a small loss of *ca.* 4%, 3.5% and 9% of linalool, carvarcrol or thymol respectively when incorporated into the coatings. This loss is insignificant and might be attributed to the low temperature used during the coating process. The concentration of linalool, carvacrol or thymol on a weight basis of dry film was 0.48, 1.43 and 2.38% (w/w) for the starch-based films containing formulation concentrations of 1, 3 and 5% (w/w) AM agents in the coatings.

In relation to the migration experiments, a first-order model described satisfactorily the kinetics of the overall release of linalool, carvacrol and thymol from the TPS heat pressed and MC-HPMC coated starch-based films. The results suggest that linalool, carvacrol and thymol incorporated into TPS starch-based films or a MC-HPMC coating is readily released into isooctane. The short-term and long-term diffusion models also adequately describe the migration of these AM agents. The results further suggest that an increase in temperature has a significant effect on the migration into isooctane from each of the AM agents in either the pressed starch-based films or the MC-HPMC coatings. The rate constant determined from the kinetic model showed an average increase of *ca.* 84% and 94% for the TPS films and MC-HPMC coatings respectively, when the temperature was increased from 15 to 35°C. The diffusion coefficients and the rate constants determined from the diffusion model and the overall kinetics analyses increased with an increase in the temperature. The average diffusion coefficients increased by *ca.* 120% and 170% from TPS films and MC-HPMC coatings matrix respectively when the temperature was increased from 15 to 35°C. An Arrhenius relationship was found to describe well, in all cases, the relationship between the

diffusion coefficient and temperature for the activation energy. The high efficiency of the release of linalool, carvacrol or thymol from starch-based films points to the great potential of these systems for AM packaging of food products to extend their shelf life and reduce the risk of food-borne illness associated with microbial contamination.

It is evident that when left in the open air without wrapping, the APTPS starch-based films containing carvacrol demonstrated a loss of *ca.* 30% after 28 days of storage. In film samples wrapped in aluminium foil only *ca.* 6% of the carvacrol was lost to the atmosphere after 28 days of storage. As expected, storage at ambient conditions of AM films wrapped in aluminium foil had a significant effect on the retention of carvacrol also in the MC-HPMC coatings. The significant retention of carvacrol in the AM films when wrapped with aluminium foil demonstrates the necessity of utilising foil in any proposed commercial application of such films in the future.

By using the *in vitro* experiments, the agar diffusion method and/or modified atmosphere methods provide preliminary information about AM activity of AM agents before being applied to real foodstuffs. In the solid media using the agar diffusion method, all of the AM films formed clear zones of inhibition against *S. aureus* and *S. cerevisiae*. Furthermore, starch-based films containing linalool, carvacrol or thymol in their coatings effectively inhibited the growth of *A. niger* on solid media in accordance with the modified microatmosphere method. The inhibitory effect of these AM agents against *S. aureus, S. cerevisiae* and *A. niger* was found to be dependent on the concentration of the AM agent in the surface coatings. Thymol was found to be the most effective AM agent, followed by carvacrol and linalool, when used with a starch-based

material as the substrate. The concentration dependence of the effectiveness was found to be linear at the higher concentrations of the AM agents (above *ca.* 0.48% (w/w)) and can be used as a measure of the sensitivity of the test microorganism to the AM agents.

The results of the challenge test experiments suggest that all the AM films containing linalool, carvacrol or thymol effectively inhibited the growth of *S. aureus*, *S. cerevisiae* and *A. niger* on the surface of Cheddar cheese and the inhibitory effect was found to be dependent on the concentration of the AM agent in the surface coating. Similarly to the results of solid media, thymol demonstrated the highest AM inhibitory effect on Cheddar cheese, followed by carvacrol and linalool. In contrast to the results obtained using the solid media, the inhibitory effect on the surface of the cheese sample was found to be linear across the entire concentration range studied, namely between zero and 2.38% (w/w), for all the AM agents in the film coatings.

5.2 The Significance of the Findings

Consumers' demand for the provision of fresh, natural foods containing minimum amounts of preservatives and that are of high quality and extended shelf-life has been increasing in recent years. Likewise, the development of renewable packaging materials is of increasing priority. Therefore, the application of biobased materials coated and/or incorporated with natural AM compounds becomes of an increasing interest and relevance in the food industry for controlling food spoilage as well as enhancing microbial safety of food products. The present study has demonstrated that starch-based materials have useful food packaging properties. The incorporation of linalool, carvacrol or thymol into such materials enhances the potential of starch-based materials

for AM packaging applications of food products by inhibiting several microorganisms of concern.

5.3 Recommendations for Further Research

It was found that the AM films derived from TPS material are best suited for packaging of food products with low a_w and at low RH levels while the APTPS materials could be used for packaging of certain foodstuffs at most a_w levels. However, the study is not complete and the following future studies are recommended to possibly enable the commercial implementation of these AM systems.

- To investigate the AM activity of TPS AM films studied in the current work against other microorganisms that are related to spoilage and safety concerns in the diary/cheese industry
- To further explore the inhibitory effect of linalool, carvacrol or thymol at lower concentrations than those used in the present study in order to identify systems that could be commercially optimal. Studies on the effects of concentration levels on the organoleptic properties of cheese samples would be valuable
- Further investigations on the effects of time, temperature and RH on the loss of AM agents from the starch-based films to the atmosphere would enable the AM efficacy of these systems to be assessed after prolonged periods of storage
- To further explore more analytical techniques such as NIR, GCMS or GCFID for accurate data acquisition and analysis in order to identify and quantify AM agents in AM films during the long term storage conditions. Studies on the data validation on

these techniques would be important in order to obtain analytical features such as limit of detection, limit of quantification, linear range, robustness of the procedure and accuracy that are required for Quality Assurance

- Further investigate the effects of AM agents incorporated into starch-based films against spoilage or pathogenic microorganisms on various other foodstuffs such as other hard cheeses, processed meats, pastrami and bakery
- To further investigate the potential for incorporating other natural AM agents such as eugenol, cinnamaldehyde, nisin, etc) into starch-based films.
- The WVP and WVTR values of TPS films obtained in this study are relatively high. This could be further explored in the future by comparing the WVP and WVTR values with typical synthetic based films such as those derived from petrochemical sources.
- To further investigate the diffusion coefficients of AM agents in the AM films at low temperatures such as (4°C).
- Since the AM agents at high concentration might have negative effects on sensory properties of the packaged foodstuffs, the effects of AM agents on the sensory properties of packaged cheese need to be explored.

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Appendix A - Properties of Polymers and AM Agents

Table A.1. Properties of methylcellulose

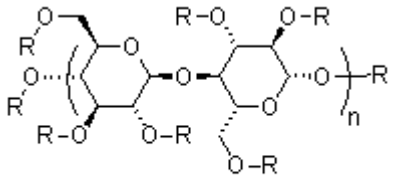
Methylcellulose	
Structure:	 R = CH_3 or H
Features:	Dissolves in water, undergoes reversible gelation upon heating, non-ionic, does not complex with ionic species and is surface active and enzyme resistant. Solutions are pseudoplastic.
Extent of Labelling:	~30 wt. % Methoxy
Molecular Weight:	Average M_n ~17,000
CAS Number:	9004-67-5
Surface Tension:	53-59 × 10^{-5} N cm^{-1} (at 25°C, 0.05 wt. %)
Viscosity:	25 mPa s (2 % in H_2O, at 20°C) (lit.)

Table A.2. Properties of hydroxypropyl methylcellulose

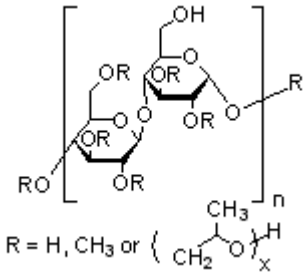
Hydroxypropyl methylcellulose	
Structure:	
Features:	Dissolves in water, undergoes reversible gelation upon heating, non-ionic, does not complex with ionic species and is surface active and enzyme resistant. Solutions are pseudoplastic.
Extent of Labelling:	1.8-2.0 mol methoxy per mol cellulose (D.S.) 29 wt. % methoxy 0.2-0.3 mol propylene oxide per mol cellulose (M.S.) 7 wt. % propylene oxide
Molecular Weight:	Average M_n ~11,500
CAS Number:	9004-65-3
Surface Tension:	43-55 × 10^{-5} N cm^{-1} (at 25°C, 0.05 wt. %)
Viscosity:	40-60 mPa s (2 % in H_2O, at 20°C)
Transition Temperature:	Gel point 58-64°C

Table A.3. Properties of the AM agent: linalool

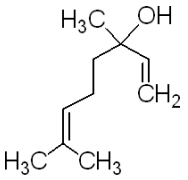
Linalool	
Product Code	L2602
Assay	≥97%
Company	Sigma-Aldrich Pty, Ltd.
Synonyms	5(±)-3,7-Dimethyl-1,6-octadien-3-ol (±)-3,7-Dimethyl-3-hydroxy-1,6-octadiene (±)-Linalool
Structure	
Molecular Formula	(CH3)2C=CHCH2CH2C(CH3)(OH)CH=CH2
Molecular Weight	154.25
CAS Number	78-70-6
Beilstein Registry Number:	1721488
FEMA Number	
Council of Europe Number	2011344
Physical State	Colourless liquid
Vapour Pressure	0.17 mm Hg (25°C)
Boiling Point	196-198°C 720 mm Hg
Flash Point	78°C
Density	0.861 g mL^{-1}
Solubility	Relatively insoluble in water but soluble in alcohol and ether.

Table A.4. Properties of AM agent: carvacrol

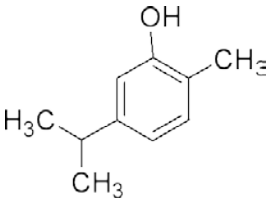
	Carvacrol
Product Code	W224502
Assay	≥98%
Company	Sigma-Aldrich Pty, Ltd.
Synonyms	5-Isopropyl-2-methylphenol; 2-p-Cymenol; 2-Hydroxy p-cymene; 2-Methyl-5-isopropylphenol; Isothymol; o-Cresol
Structure	
Molecular Formula	$(CH_3)_2CHC_6H_3(CH_3)OH$
Molecular Weight	150.22
CAS Number	499-75-2
FEMA Number	2245
Council of Europe Number	
Appearance/Physical State	Colourless to pale yellow liquid
Vapour Pressure	0.0232 mmHg (25 °C)
Boiling Point	236-237°C
Melting Point	3-4°C
Flash Point	106°C
Density	0.976 g mL^{-1} at 20°C
Solubility	Relatively insoluble in water but soluble in alcohol and ether.

Table A.5. Properties of the AM agent: thymol

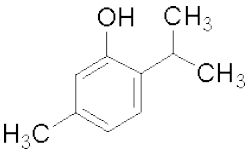
	Thymol
Product Code	TO501
Assay	≥99.5%
Company	Sigma-Aldrich Pty, Ltd.
Synonyms	2-Isopropyl-5-methylphenol; 5-Methyl-2-isopropylphenol; 5-Methyl-2-(1-methylethyl) phenol; m-Cresol, p-Cymene-3-ol; p-Cymene, 3-Hydroxy; Isopropyl-cresol; m-Thymol, Thyme camphor
Structure	
Molecular Formula	2-[$(CH_3)_2CH$]C_6H_3-5-(CH_3)OH
Molecular Weight	150.22
CAS Number	89-83-8
FEMA Number	
Council of Europe Number	
Appearance/Physical State	White crystals to powder
Vapour Pressure	0.0022 mm Hg (25°C), 1 mm Hg (64°C)
Boiling Point	232°C
Melting Point	49-51°C
Flash Point	110°C
Density	0.965 g mL^{-1}
Solubility	Slightly soluble in water and glycerol, very soluble in alcohol and in ether, freely soluble in essential oils and in fatty oils

Table A.6. Characteristics and growth conditions of *Staphylococcus aureus*, *Saccharomyces cerevisiae* and *Aspergillus niger*

	Bacteria	**Yeast**	**Fungi**
Genus	*Staphylococcus*	*Saccharomyces*	*Aspergillus*
Species	*Aureus*	*cerevisiae*	*Niger*
Collection Acronym	UNSW*	UNSW	UNSW
Accession Number	056201	703100	809000
Equivalent to	NCTC[†] 10652 ATCC[≠] 13565		ATCC 16404
Isolated/Derived from	Lyophilised culture	Fermenting prunes	Blueberry, North Carolina
Growth Medium	Nutrient agar	Malt agar	Malt agar
Incubation Temperature	37°C	25°C	25°C
Incubation Time	24 h	48 h	1 week
Growth conditions	Aerobic	Aerobic	Aerobic
Special Features and Usage	Type strain	Non-flocculating	Assay of AM agents and AM preservatives

*UNSW = Culture collection, University of New South Wales, Sydney, Australia.

[≠]ATCC =American Type Culture Collection

[†]NCTC = The National Collection of Type Cultures, United Kingdom.

Appendix B – Supplementary Figures Pertaining to the Properties of the Starch-based Materials

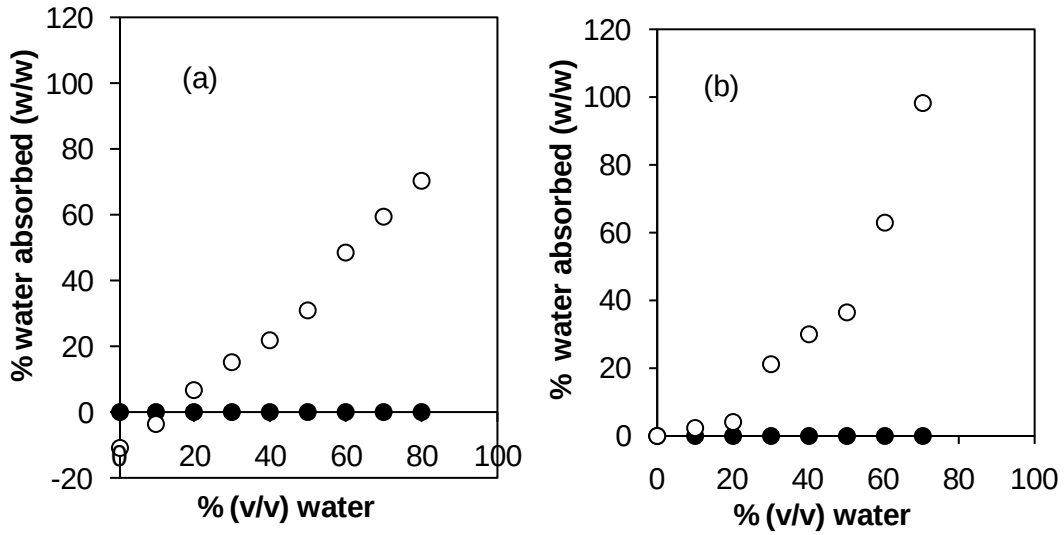


Figure B.1. Percent water uptake of TPS (○) and APTPS (●) films at 20 ± 1°C as a function of: (a) ethanol concentration in the ethanol/water and (b) glycerol concentration in the glycerol/water immersion mixture after 5 min immersion.

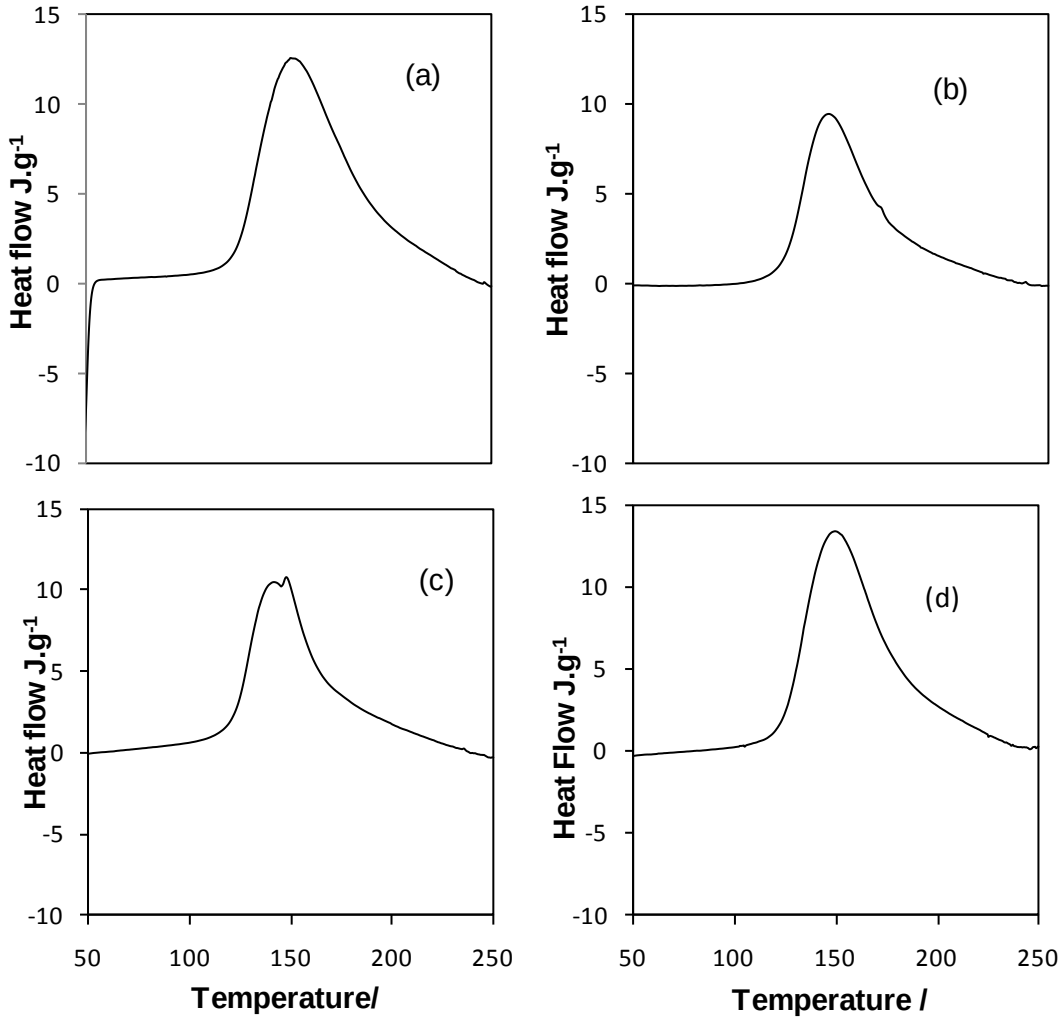


Figure B.2. DSC thermograms of: (a) control TPS film and (b) TPS film containing 1.04% (w/w) linalool (c) TPS film containing 1.1% (w/w) carvacrol (d) TPS film containing 1.2% (w/w) thymol.

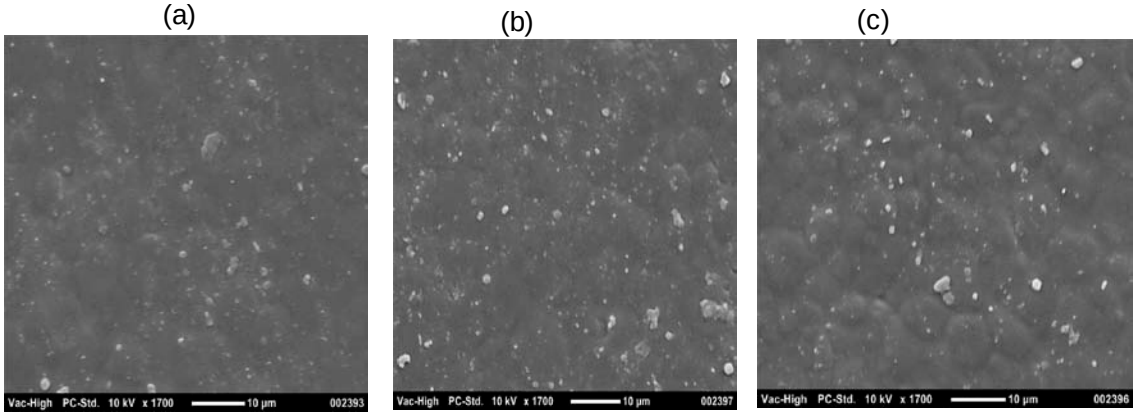


Figure B.3. SEM micrographs of: (a) TPS film containing 0.7% (w/w) linalool (b) TPS film containing 1.0% (w/w) linalool and (c) TPS film containing 2.9% (w/w) linalool

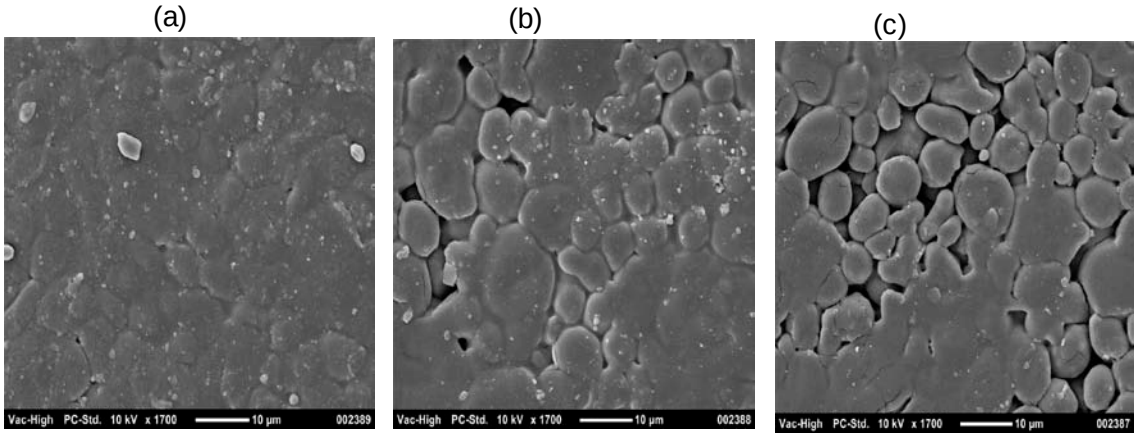


Figure B.4. SEM micrographs of: (a) TPS film containing 0.9% (w/w) thymol (b) TPS film containing 1.2% (w/w) thymol and (c) TPS film containing 3.8% (w/w) thymol

Appendix C - Supplementary Figures Pertaining to the Migration of Antimicrobial Agents into Food Simulants

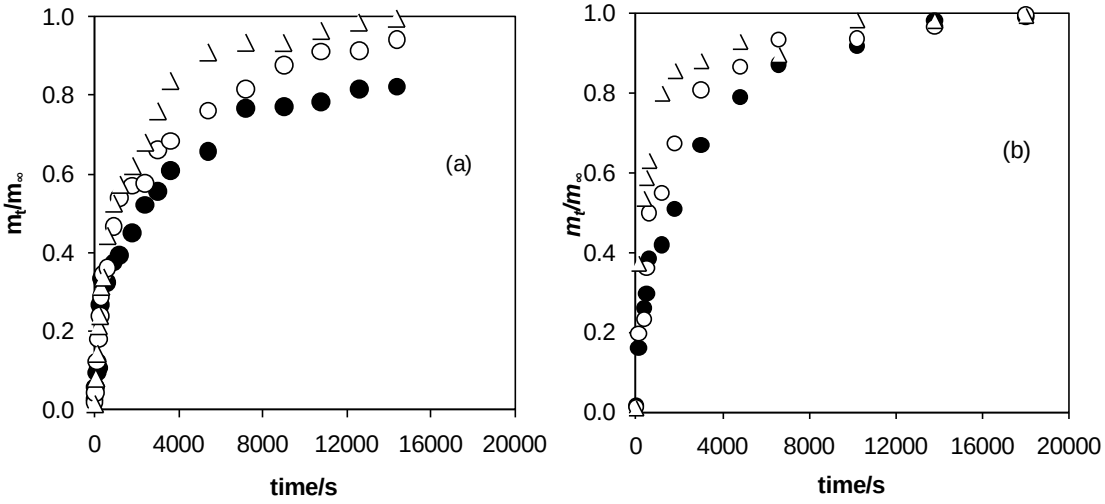


Figure C.5. Plot of the mass fraction m_t/m_∞ of linalool released into isooctane versus *t* at (●) 15, (○) 25 and (Δ) 35°C from: (a) heat pressed TPS and (b) MC-HPMC coated starch-based film.

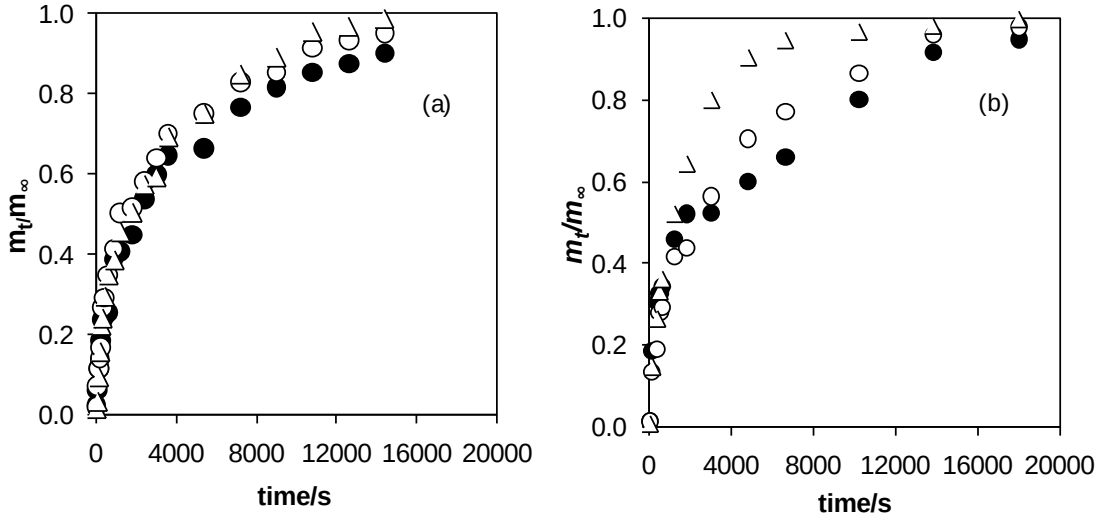


Figure C.6. Plot of the mass fraction m_t/m_∞ of thymol released into isooctane versus t at (●) 15, (○) 25 and (Δ) 35°C from: (a) heat pressed TPS and (b) MC-HPMC coated starch-based film.

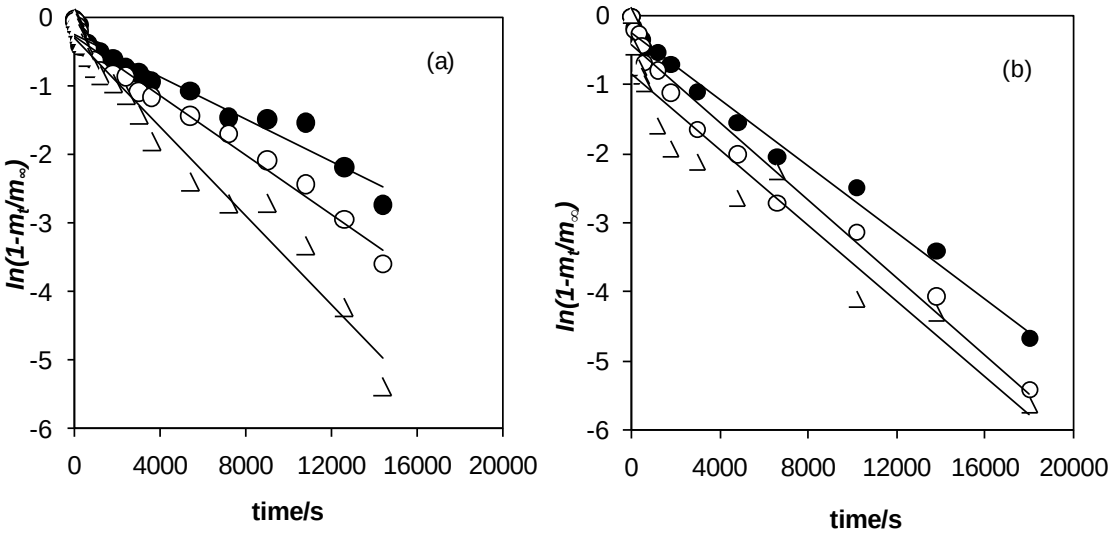


Figure C.7. Plots of ln(1 - m_t/m_∞) versus t for the migration of linalool into isooctane at (●) 15, (○) 25 and (Δ) 35°C from: (a) heat pressed and (b) MC-HPMC coated starch-based film.

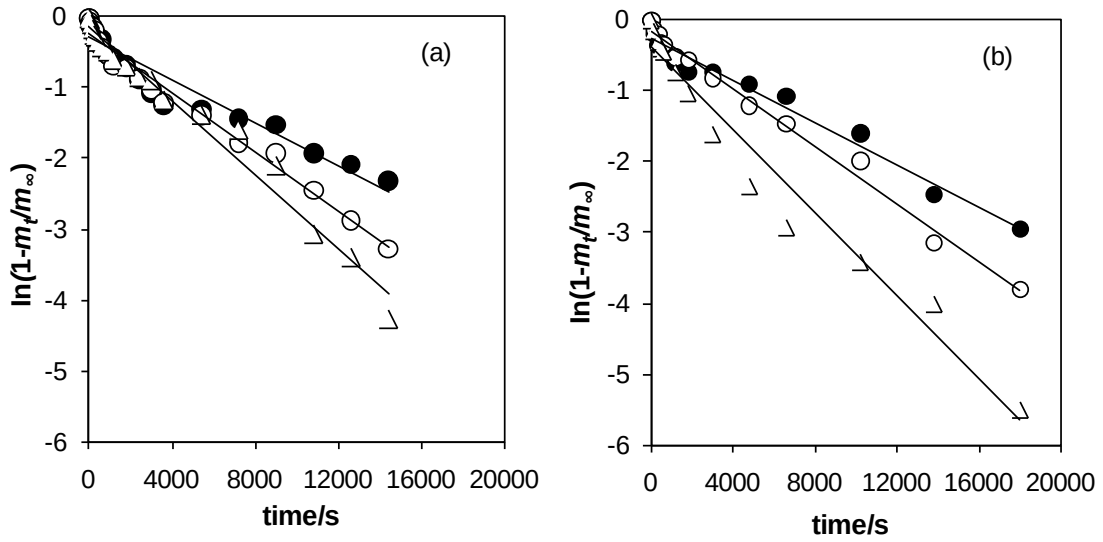


Figure C.8. Plots of ln(1 - m_t/m_∞) versus t for the migration of thymol into isooctane at (●) 15, (○) 25 and (Δ) 35°C from: (a) heat pressed and (b) MC-HPMC coated starch-based film.

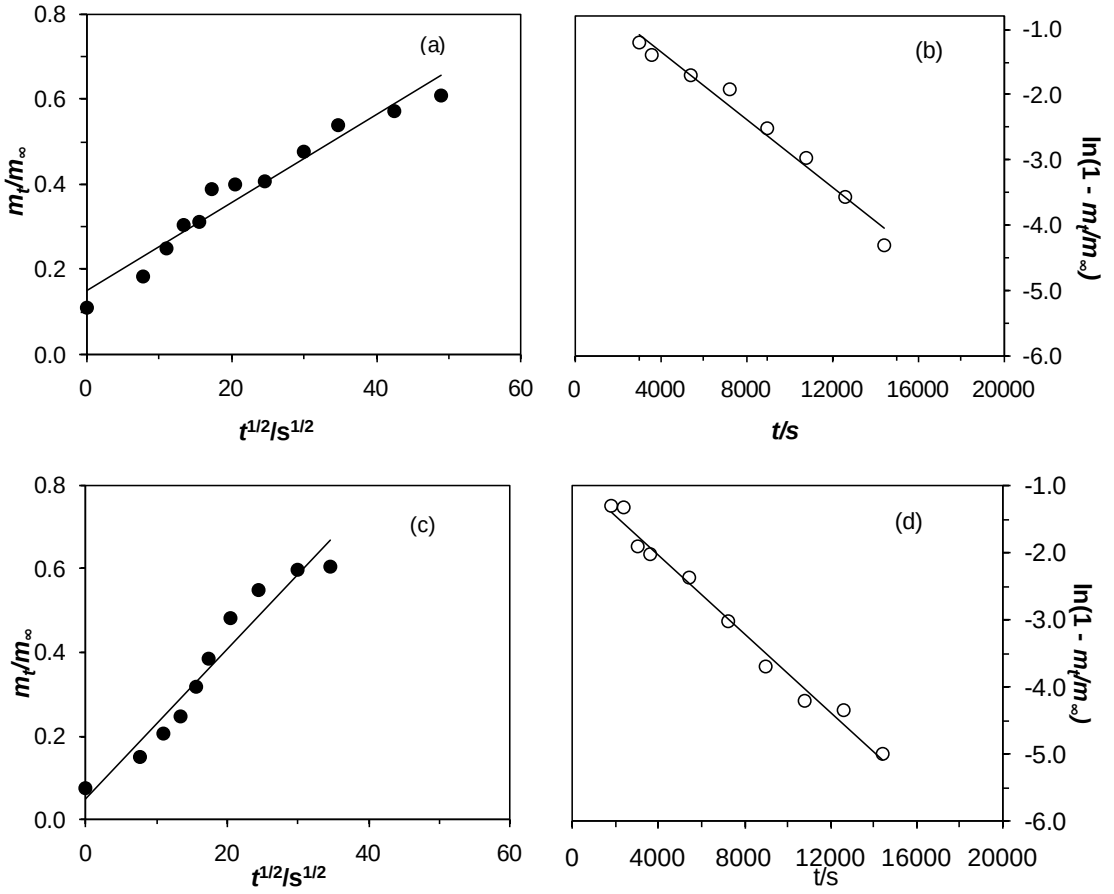


Figure C.9. Plots of: (●) m_t/m_∞ versus $t^{½}$ and (○) ln(1 - m_t/m_∞) versus t for the migration of carvacrol from heat pressed starch-based film into isooctane at: (a-b) 15°C and (c-d) 35°C.

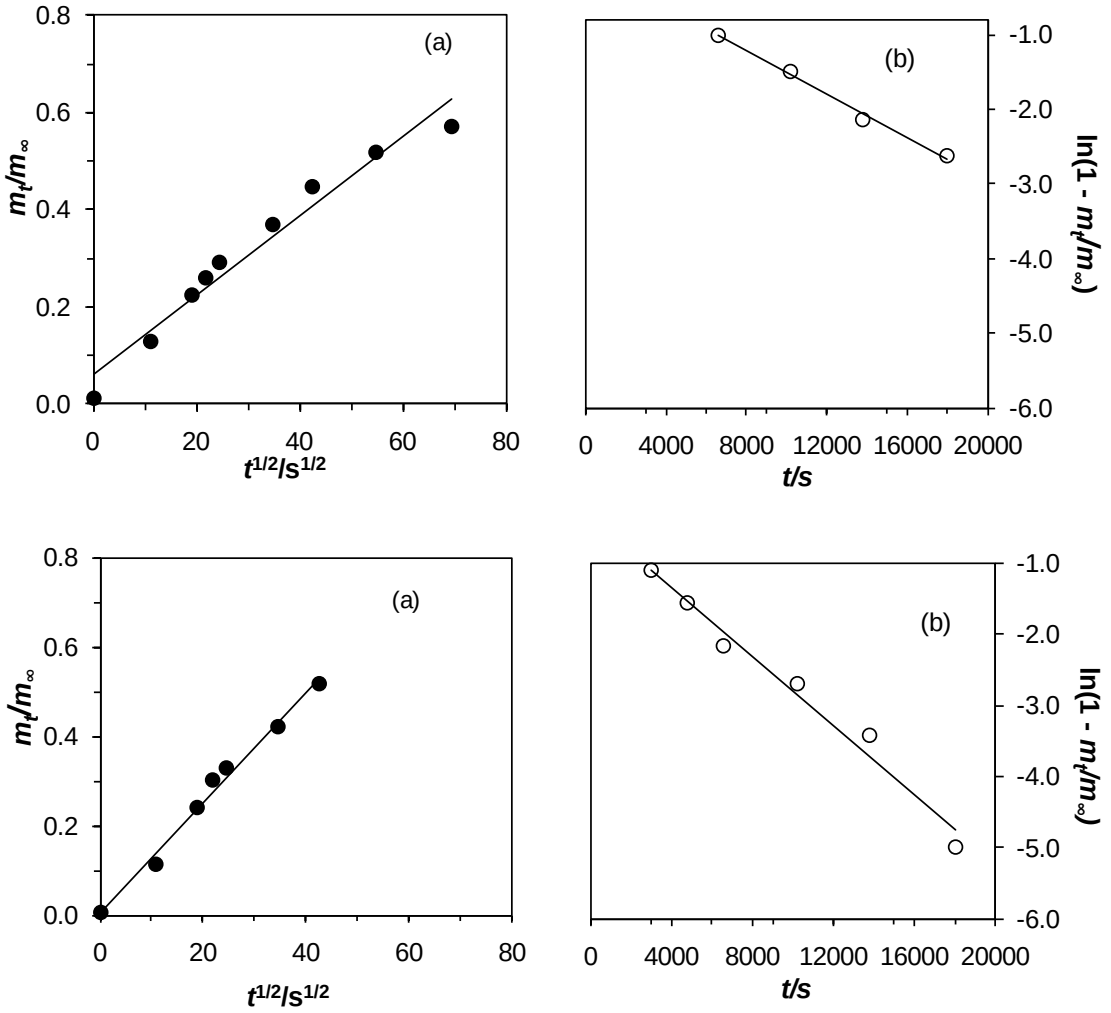


Figure C.10. Plots of: (●) m_t/m_∞ versus $t^{½}$ and (○) ln(1 - m_t/m_∞) versus t for the migration of carvacrol from MC-HPMC coated starch-based film into isooctane at: (a-b) 15°C and (c-d) 35°C.

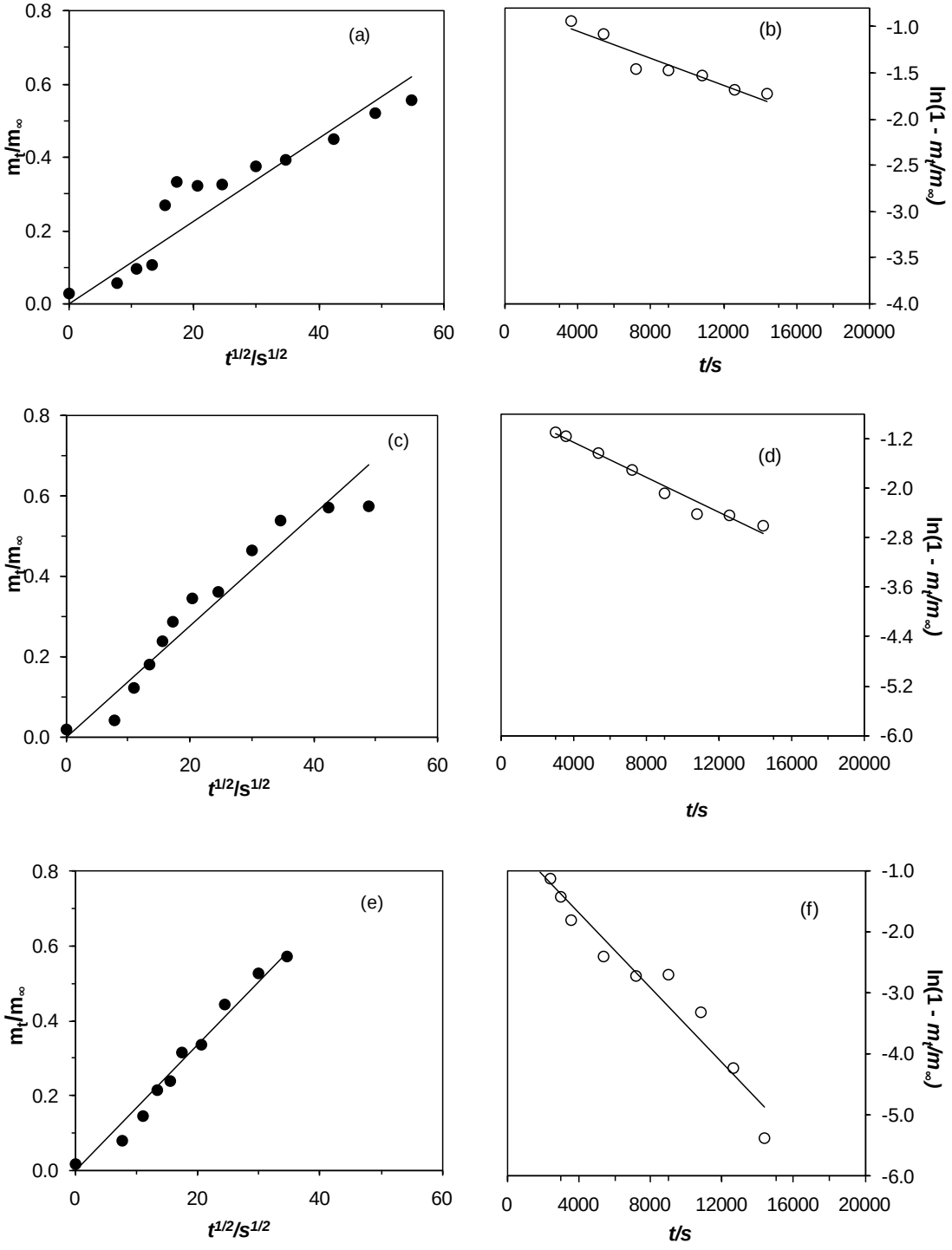


Figure C.11. Plots of: (●) m_t/m_∞ versus $t^{½}$ and (○) ln(1 - m_t/m_∞) versus t for the migration of linalool from heat pressed starch-based film into isooctane at: (a-b) 15°C, (c-d) 25°C and (e-f) 35°C.

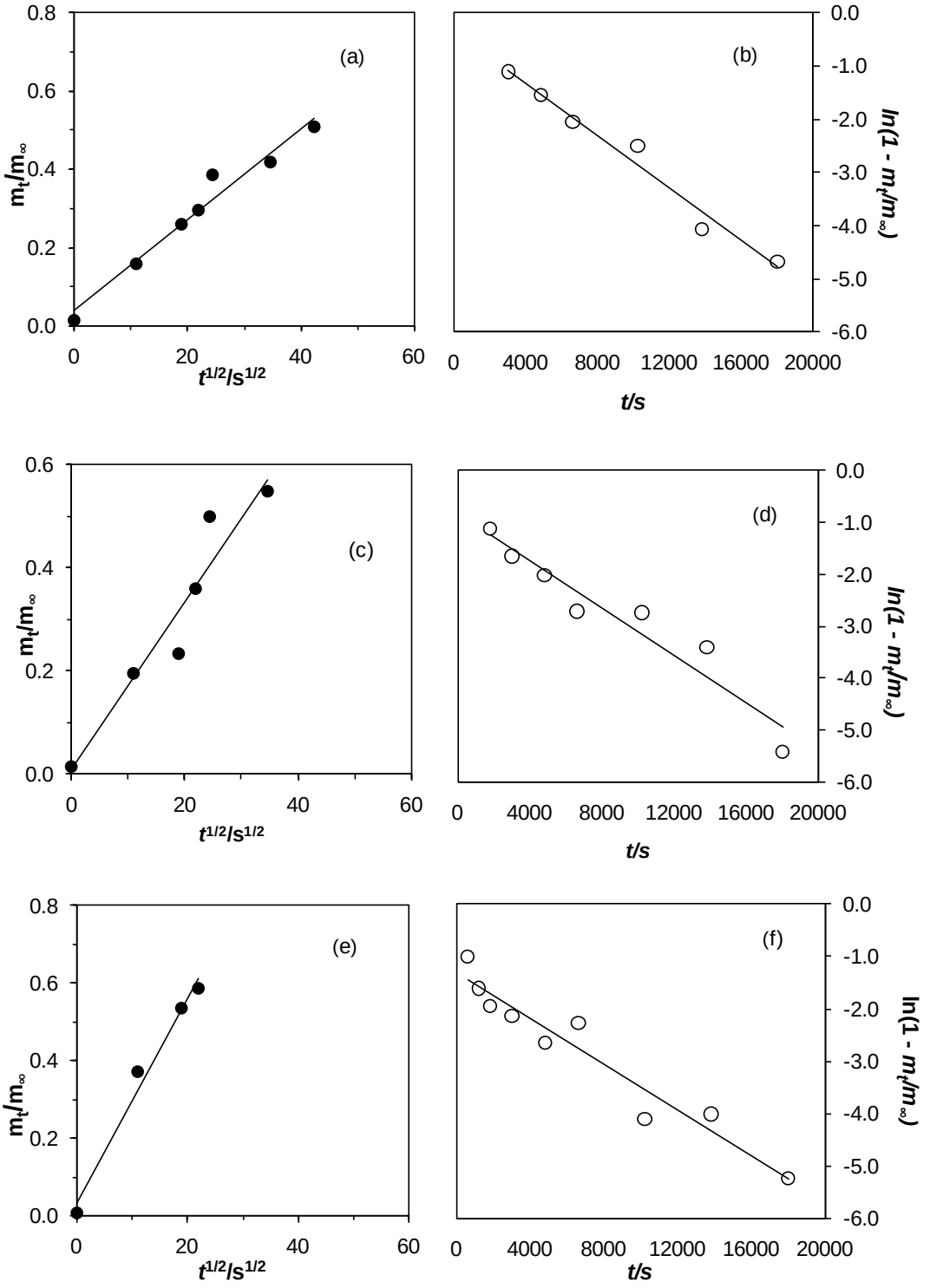


Figure C.12. Plots of: (●) m_t/m_∞ versus $t^{½}$ and (○) ln(1 - m_t/m_∞) versus t for the migration of linalool from MC-HPMC coated starch-based film into isooctane at: (a-b) 15°C, (c-d) 25°C and (e-f) 35°C.

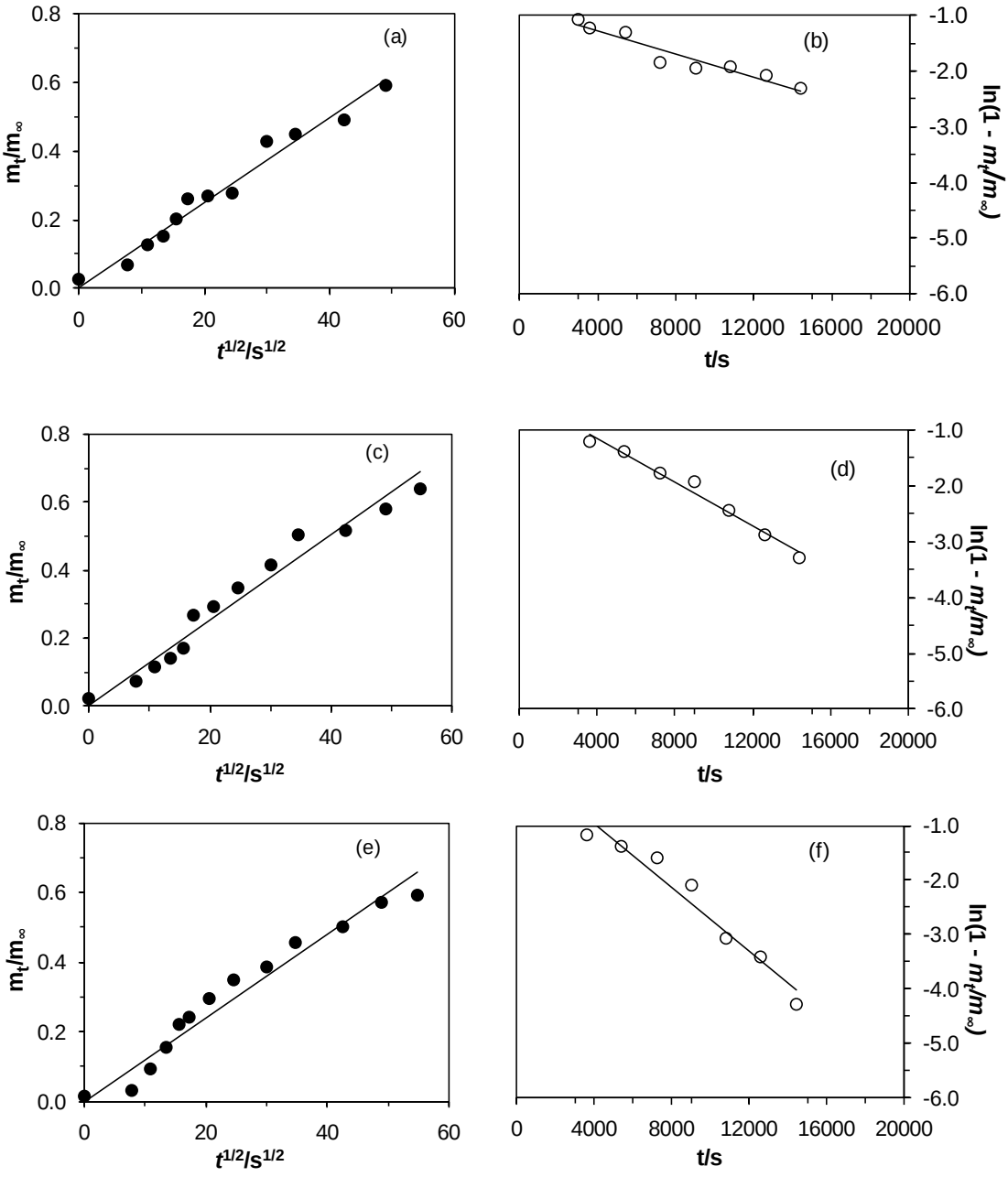


Figure C.13. Plots of: (●) m_t/m_∞ versus $t^{½}$ and (○) ln(1 - m_t/m_∞) versus t for the migration of thymol from heat pressed starch-based film into isooctane at: (a-b) 15°C, (c-d) 25°C and (e-f) 35°C.

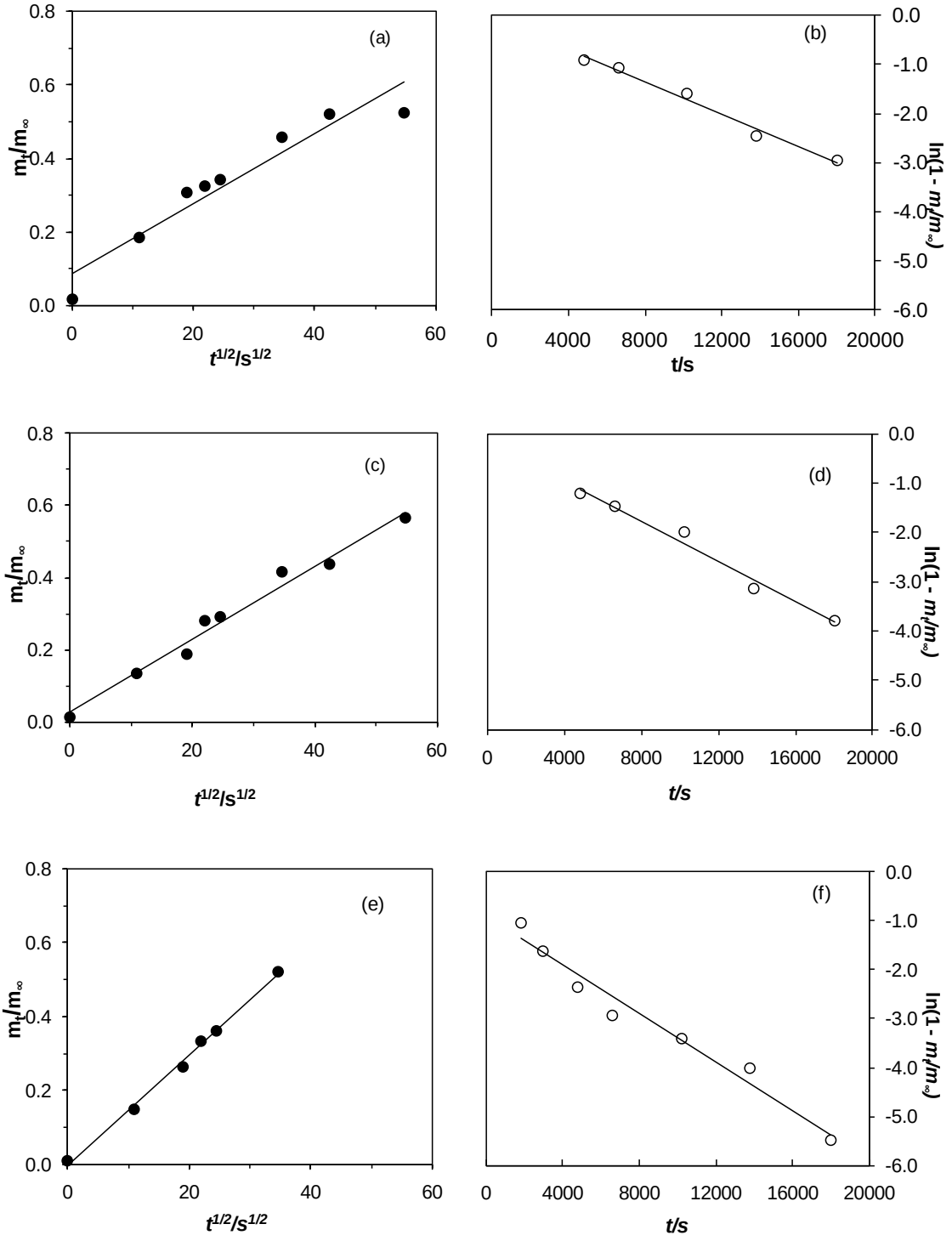


Figure C.14. Plots of: (●) m_t/m_∞ versus $t^{½}$ and (○) ln(1 - m_t/m_∞) versus t for the migration of thymol from MC-HPMC coated starch-based film into isooctane at: (a-b) 15°C, (c-d) 25°C and (e-f) 35°C.

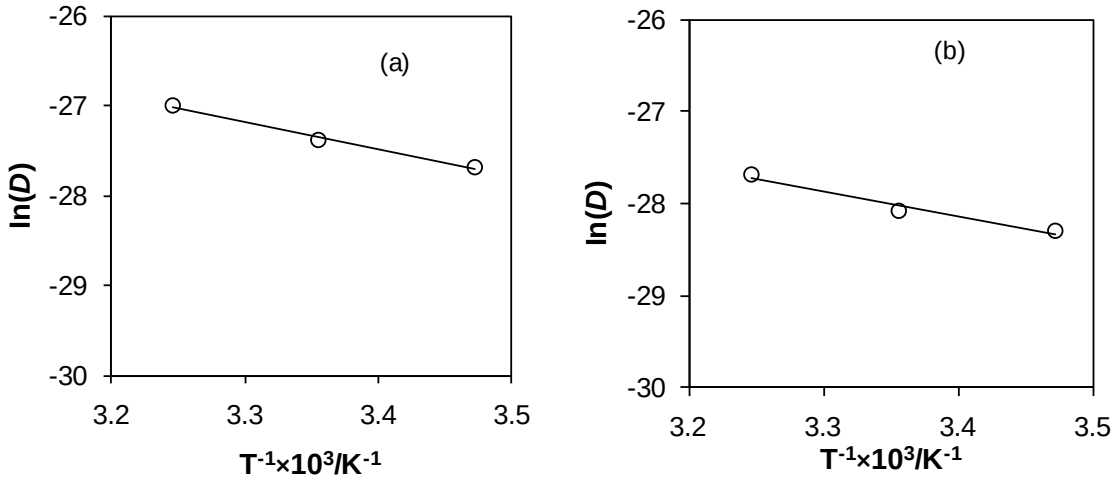


Figure C.15. Arrhenius plots of ln(D) versus 1/T for the release of linalool into isooctane from: (a) the heat pressed and (b) MC-HPMC coated starch-based films.

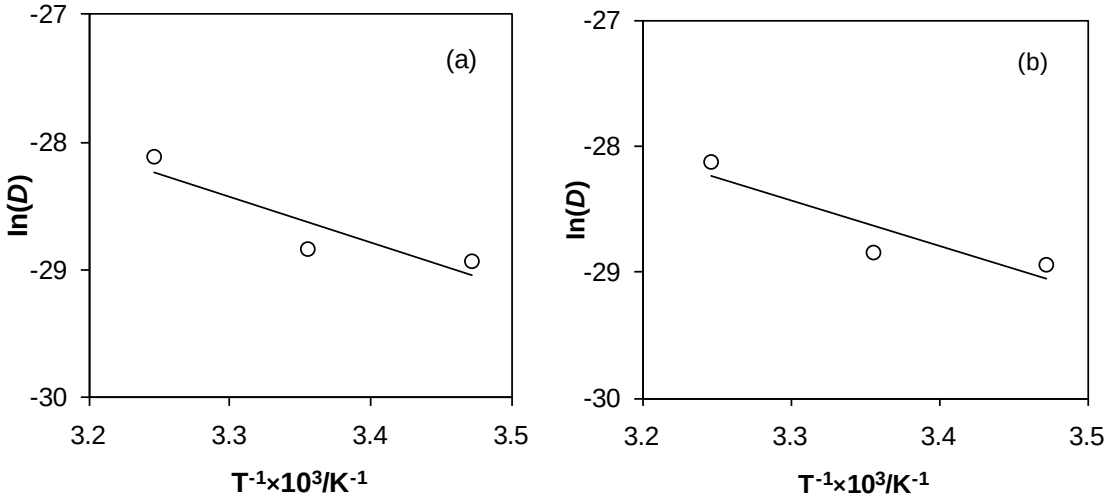


Figure C.16. Arrhenius plots of ln(D) versus 1/T for the release of Thymol into isooctane from: (a) the heat pressed and (b) MC-HPMC coated starch-based films.

Appendix D - Supplementary Figures Pertaining to Antimicrobial Activity of coated APTPS on Solid Media

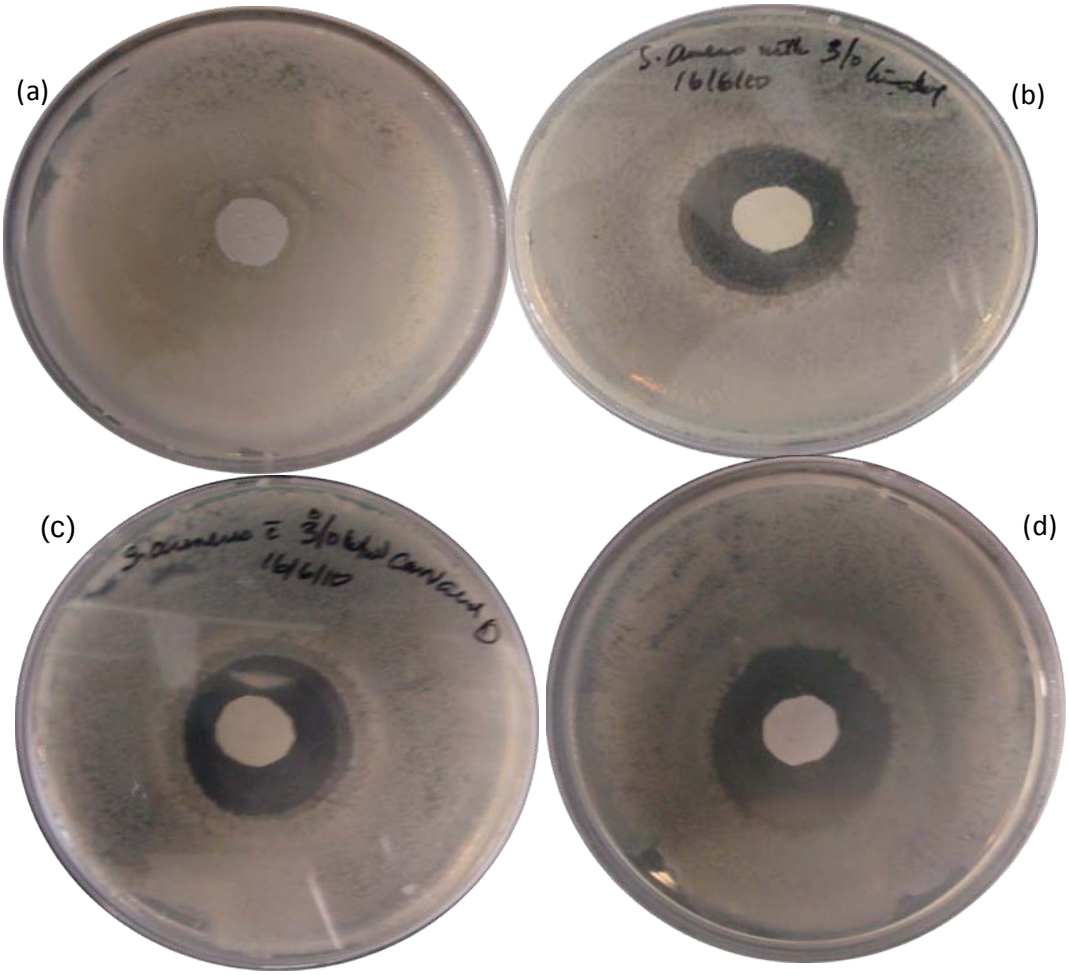


Figure D.17. Inhibition of *S. aureus* on solid media at 37°C after 24 h on starch-based coated films containing: (a) no AM agent, (b) 1.43% (w/w) linalool, (c) 1.43% (w/w) carvacrol, and (d) 1.43% (w/w) thymol.

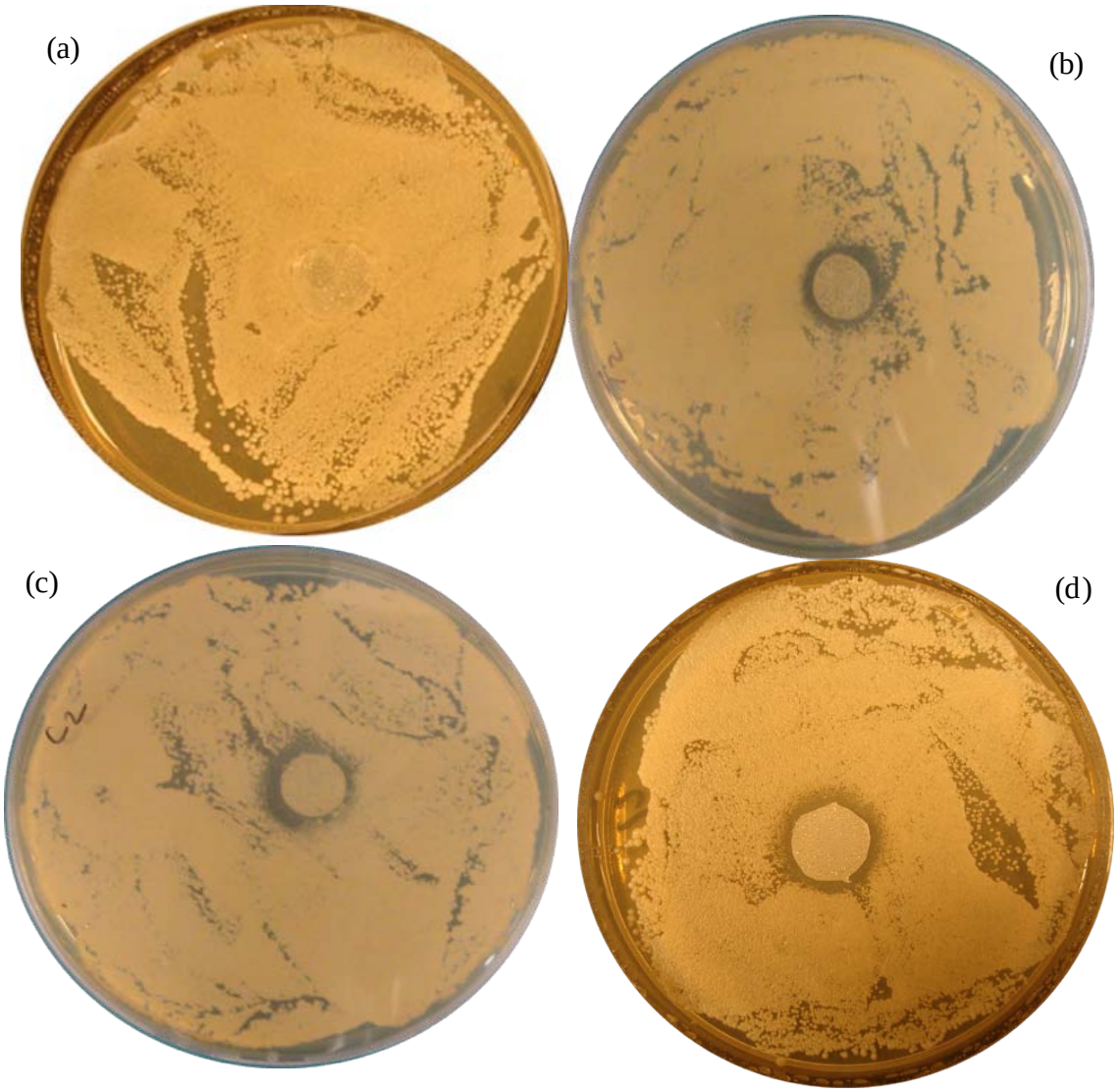


Figure D.18. Inhibition of *S. cerevisiae* on solid media by starch-based AM films coated with: (a) No AM agent (control), (b) 1.43% (w/w) linalool, (c) 1.43% (w/w) carvacrol and (d) 1.43% (w/w) thymol, after incubation for 48 h at 25°C.

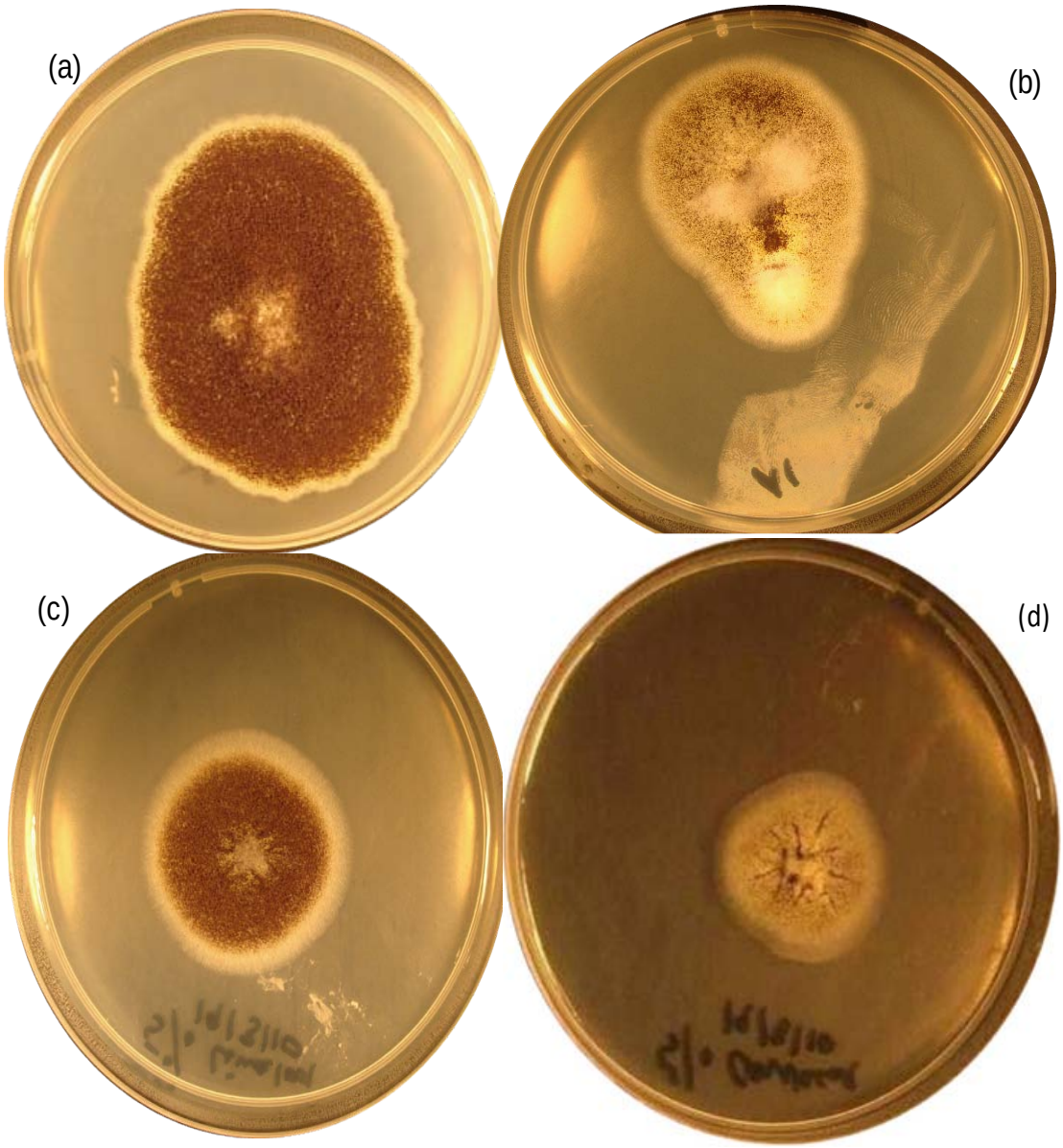


Figure D.19. Inhibition of *A. niger* on solid media at 25°C after 7 d by starch-based AM films containing: (a) The control film (contained no AM agent in its coating), (b) 1.43% (w/w) linalool, (c) 1.43% (w/w) carvacrol and (d) 1.43% (w/w) thymol in their coating.