Chapter 4

Antimicrobial Activity and Properties of Chitosan/Methylcellulose Films Incorporated with Carbendazim

4.1 Abstract

Chitosan/methylcellulose (C/MC) based film is a biopolymer material that incorporates carbendazim to provide antimicrobial properties. Carbendazim with various contents (0.8, 1.6, 3.2 and 4.8 g/100 g of solid C/MC) was blended into the C/MC film-solution. The effect of this carbendazim content on the mechanical properties, color, oxygen permeability (OP), water vapor permeability (WVP), solubility, sorption isotherms (SI), morphology and antimicrobial activity of C/MC films were then investigated. The tensile strength (TS) and percent elongation (%E) of the C/MC films with the lowest carbendazim contents (0.8 and 1.6 g/100 g solid of C/MC) were not significantly different from the control. The TS and %E of the films decreased with increasing in carbendazim levels from 3.2 to 4.8 (g/100 g of solid C/MC). The OP and WVP increased slightly with increasing carbendazim contents. The moisture sorption isotherms of C/MC films incorporated with carbendazim were examined for water activities in the range of 0.11-0.86 at 25±0.5^{o}C. These isotherms showed that carbendazim content affected the equilibrium moisture content (%EMC) of the films and the %EMC of all films dramatically increased above a_w=0.66. The C/MC films incorporated with carbendazim exhibited lower %EMC than that of the control films under all relative humidity conditions. Moreover, the morphology of the films was examined using a scanning electron microscope (SEM) and the morphology of films containing increased levels of carbendazim became rougher and less homogenous. The inhibitory effect of the C/MC films incorporating carbendazim on yeast (*Saccharomyces cerevisiae*) and fungi (*Aspergillus oryzae*) growth was also investigated. Our results show that the C/MC film incorporated with 1.6 g carbendazim/100 g of solid C/MC was the best film for potential use in extending the shelf life of fruit and food products.

4.2 Introduction

The shelf life of fruits, vegetable and foods in general easily reduce by many factors. In which, the main cause of food spoilage is the growth of microorganisms on the surface of the products. Chitosan is a biopolymer material has been used to extend the shelf life for food products; however, it must to develop the antimicrobial activity and some properties because of three mechanisms. In the first one, chitosan is the second most abundant polysaccharide on earth and has an antimicrobial effect on microorganism growth (Goldberg *et al.*, 1990). The positive charges present in the polymeric chain of chitosan interact with negative charges from the residues macromolecules in the microbial membranes causing disruption and cell death (Helander *et al.*, 2001; Young and Kauss, 1983).

The second mechanism concerns on physical – chemical properties of chitosan. Chitosan films have good mechanical and oxygen barrier properties (Caner *et al.*, 1998). However, chitosan exhibits a poor tensile strength when wet and is rigid with a high elastic modulus and poor elongation properties (García *et al.*, 2004; Sangsuwan *et al.*, 2008b). Blending methylcellulose (MC) with chitosan can create the flexible film and the flexibility increase with increasing MC content (García *et al.*, 2004). MC films are also selectively permeable to CO_2 and O_2, and hence, retard the respiration and ripening of many fruits and vegetables by limiting the availability of O_2 (Park *et al.*, 1993). On the other hand, moisture sorption isotherms are an important property to evaluate the effect of relative humidity (RH) on film performance (Cadden, 1988; Lai and Padua, 1998). Since the addition or removal of water may cause phase transitions in the macromolecular structure (Schwartzberg, 1986; Torres, 1994), knowledge of sorption isotherms is important for predicting water sorption properties of hydrophilic films, stability and quality changes during packaging and storage of intermediate and high moisture foods (Kulchan *et al.*, 2010).

The third mechanism indicates that the application antimicrobial agent on chitosan/methylcellulose (C/MC) film should be developed. The incorporation of antimicrobial agents into chitosan films, create an environment inside the package that may delay or prevent the growth of microorganisms on the product's surface and lead to an extension of its shelf-life (Cha and Chinnan, 2004). Adding benzoate and sorbate into C/MC films to improve antimicrobial activity has also been reported

(Chen *et al.*, 1996). Essential plant oils, including anise, basil, coriander, oregano, garlic oil, potassium sorbate and bacteriocin (nisin) into chitosan films and C/MC films as an supplemental antimicrobial activity agents were reported (Pranoto *et al.*, 2005; Sangsuwan *et al.*, 2012; Zivanovic *et al.*, 2005). The activity of antimicrobial films has been tested against *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Listeria monocytogenes and Bacillus cereus.* The results showed that chitosan film had no inhibitory effect. However, the incorporation of 100 µl of garlic oil/g, 100 mg potassium sorbate/g or nisin at 51,000 IU/g of chitosan did provide antimicrobial activity against *Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus* (Pranoto *et al.*, 2005; Zivanovic *et al.*, 2005).

Carbendazim (methyl-2-benzimidazole carbamate - $C_9H_9N_3O_2$) is a widely used broad-spectrum benzimidazole fungicide that plays a very important role in plant disease control. Carbendazim solutions have been used directly to control of fungi and diseases of plants and fruits in postharvest food storage, in seed pre-planting treatment, and as a fungicide in paint, paper and wood (Clemons and Sisler, 1971; López-Herrera and Zea-Bonilla, 2007). Blending carbendazim into C/MC film to create an antimicrobial environment on the surface material is a new trend in fruits and food packaging. There are no reports on the effect of carbendazim concentration on the properties of C/MC based films.

Therefore, the objectives of this study were to evaluate some aspects of C/MC films with carbendazim incorporated including mechanical properties, WVP, OP, morphology and antimicrobial activity. Knowledge of these results is important for applications of antimicrobial films for fruit storage.

4.3 Materials and Methods

4.3.1 Materials

Chitosan with a degree of deacetylation of 90% and purity >99.75% was purchased from Bannawach Bio-line Co. Ltd., Thailand; methylcellulose (viscosity 10-25 mP.s, Methocel # 64605) was purchased from Sigma-Aldrich, USA; PEG 400 was purchased from Vichavit, Thailand; carbendazim (50%w/v) was purchased from

BaSF Co., Thailand, ethanol (99.8%) and acid acetic (98%) were purchased from MERCK, Germany.

4.3.2 Film preparation

The initial chitosan solution was prepared by dissolving 1.5 g of chitosan in 100 mL of 1% acetic acid solution. One gram of methylcellulose was dissolved in 100 mL of ethanol: water (1:3). Solutions of chitosan and methylcellulose were then mixed in a beaker with a stirrer. One gram of polyethylene glycol (PEG) 400 was used as a plasticizer (Sangsuwan *et al.*, 2008a). Carbendazim (active ingredient) with various contents (0.8; 1.6; 3.2 and 4.8 g/100 g solid of C/MC) was then blended into the C/MC film-solution. The solutions were then filtered, cooled and degassed. For each of the film-forming solutions, 50 mL were poured onto glass plates (14.5 cm diameter) and dried at ambient temperature for 48 to 72 hours. The films were then peeled off the glass plates and conditioned at 25±2^{o}C, 52±2% RH for at least 48 hours prior to evaluation.

4.3.3 Film thickness

The thickness of each film was determined using a digital micrometer (CE, Japan, sensitivity ± 0.01 mm) at 5 random positions on each film. The reported thickness values were the average of at least 30 measurements.

4.3.4 Opacity and color of films

Opacity and color (L*, a*, b*, chroma and hue) of films were measured using the Hunterlab color meter ColorQuest XE (The color Management Company, Reston, Virginia, USA)calibrated with a white and a black tiles. Absolute measurements were displayed as tristimulus color values which closely represents human sensitivity. Readings were taken on 8 samples for each treatment.

4.3.5 Mechanical properties

A texture analyzer (TA.XT.plus, Stable Micro Systems Company, England) was used to determine film tensile strength (TS) and percent elongation (%E)

following ASTM D638 M (ASTM, 1993). Film specimens were first cut into rectangular strips, 1 cm x 10 cm. The initial grip separation was 5 cm and the crosshead speed was 25 mm/min. The TS was calculated by dividing the peak load by the cross-sectional area (average thickness x 1cm) of the initial specimen. The %E was defined as the percent change in the length (ΔL) of the specimen to its original length (L) between the grips (5 cm). Tensile strength and percent elongation results were obtained from ten replications.

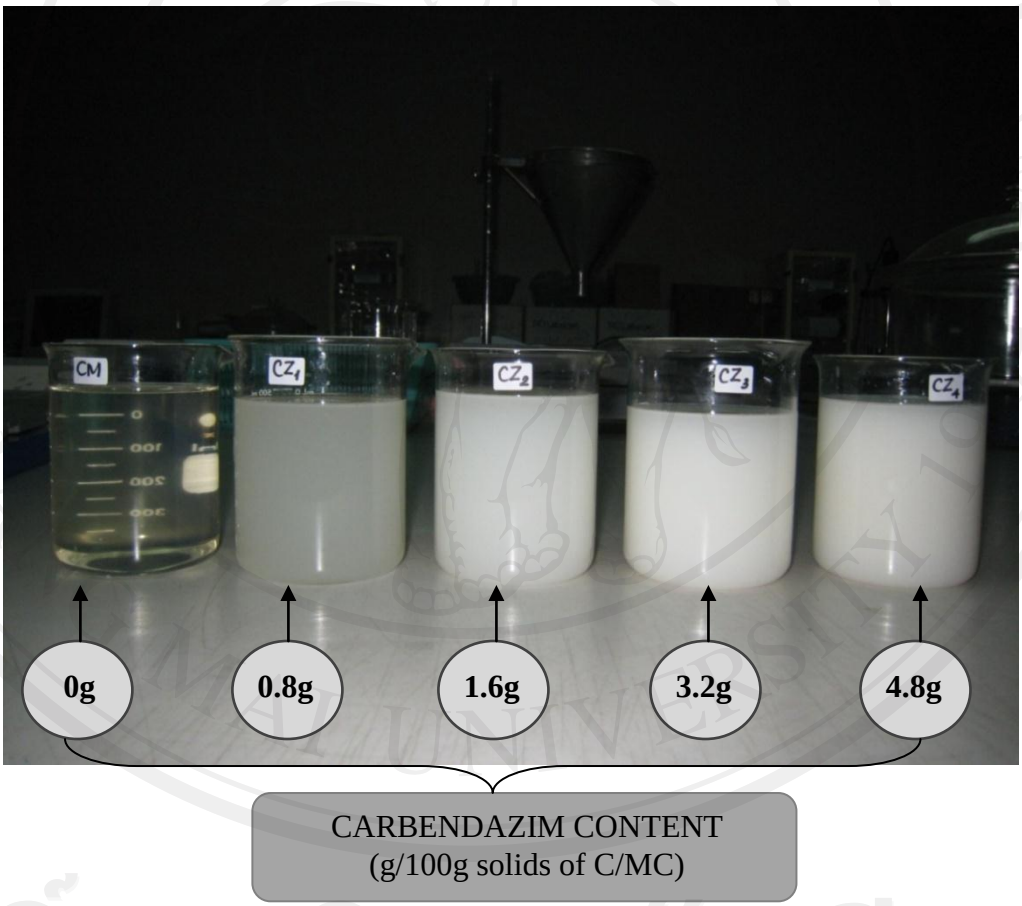


Figure 4.1 Preparing chitosan/methylcellulose solutions incorporated with different carbendazim contents.

4.3.6 Water vapor permeability

Water vapor permeability (WVP) was determined according to ASTM E96-00 (ASTM, 2000). Film specimens approximately 8 cm diameter were mounted on the aluminum cups containing 10 g of silica gel. Paraffin was used to fix a film specimen

on the wide rim of an aluminum cup (Rachtanapun and Rattanapanone, 2011). The sealed cups were then weighed and kept in desiccators with a saturated solution of magnesium nitrate salt which provided 53±1% RH and 25±1^{o}C throughout the experiment. The weight gain of the aluminum cups covered with films was recorded every 10 hours for 5 days (Turhan and Sahbaz, 2004). The test was performed in triplicate and the WVP was calculated using the following equation (4.1):

$$WVP = \frac{Ct}{A\Delta p} \qquad (4.1)$$

Where: WVP is g m/m^2 s Pa

t is the film thickness (m)

A is area of the exposed film (0.003215 m^2)

Δp is the water vapor pressure differential across the film (Pa) (the vapor pressure of pure water at 25^{o}C is 23.73 mmHg)

C is the slope of the weight gain of the cup, to nearest 0.0001 g, versus time

4.3.7 Oxygen permeability

Oxygen permeability (OP) was tested using a Gas Permeability Tester VAC-V1 (M and E Instruments, Jinan, China) according to ASTM D1434-82 (ASTM, 2003). A film specimen with 10 cm diameter was initially fixed between the upper and lower clampers. Oxygen in both chambers was then removed under vacuum for 8 hours. After that, oxygen was allowed to flow into the upper chamber. The amount of oxygen that permeated through the film in the lower chamber was then determined. The test was done in duplicate at 23 ± 1^{o}C, 0% RH (Sangsuwan *et al.*, 2008a).

4.3.8 Film solubility

Films were cut into square 1cm x 1cm pieces and dried to constant weight in a vacuum oven at 60^{o}C for 24 hours to obtain the initial film dry weight (W_d). Each film was then placed in a bottle containing 20ml distilled water for 24 hours under gentle agitation and controlled temperature at 25^{o}C. Films were then dried under the

same conditions to obtain the dry weight of water – leached film (W_{ws}). Film solubility was calculated using equation 1:

$$\% \text{ Solubility} = (W_d - W_{ws}) \times 100 / W_d \quad (4.2)$$

4.3.9 Sorption isotherms

Water sorption isotherms were determined using the method of discontinuous registration of weight changes in a static system. Moisture sorption isotherms of the chitosan/methylcellulose films incorporated with carbendazim were determined by placing film samples into a controlled humidity environment at a constant temperature until an equilibrium state was obtained.

Film specimens (30 mm x 30 mm) were pre-dried for 7 days in vacuum desiccators containing silica gel beads and then were placed at 25±2^{o}C over saturated salt solutions in separated desiccators each having the desired relative humidity conditions (11.2; 33.5; 44.5; 53.1; 66.4; 75.1 and 85.5 RH) (Table 4.1). Weights of film specimens were determined daily and when the difference between two consecutive weightings was approximately equal (not greater than 0.1% of the sample weight), it was assumed that an equilibrium condition had been reached. The moisture contents of the films were then determined by oven drying at 105^{o}C for 10 hours (Mali *et al.,* 2005). The initial moisture content of a sample at each specific relative humidity was determined using equation 2 (Rachtanapun, 2007):

$$M_i = \frac{W_e - W_D}{W_D} \times 100 \quad \text{(g/g dry solids)} \quad (4.3)$$

Where M_i is the initial moisture content of the film (g/g)

W_i is the initial weight of the film (g)

W_D is the weight of the film after drying (g)

The equilibrium moisture content was next calculated as equation 3 (Rachtanapun *et al.,* 2010).

$$M_e = \frac{W_e}{W_i} (Mi + 1) - 1 \text{ (g/g dry solids)} \quad (4.4)$$

Where M_e is the equilibrium moisture content of the film (g/g dry solids)

W_e is the equilibrium of the film (g)

W_i is the initial weight of the film (g)

M_i is the initial moisture content of the film (g/g)

Table 4.1 Relative humidity control chamber using saturated salt solutions at 25 ± 0.5°C (Rachtanapun, 2007)

Saturated salt solutions	Equilibrium (Expected) % RH	Actual reading % RH
1. Lithium Chloride	11.30	11.2±0.16
2. Magnesium Chloride	32.78	33.5±0.20
3. Potassium Carbonate	43.16	44.5±0.20
4. Magnesium Nitrate	52.89	53.1±0.19
5. Sodium Nitrate	64.00	66.4±0.17
6. Sodium chloride	75.29	75.1±0.50
7. Potassium Chloride	84.24	85.5±0.09

4.3.10 Morphology observation

The surface and cross-sectional morphologies of the C/MC films with and without carbendazim were examined using scanning electron microscopy (JEOL, JSM-5910LV, Tokyo, Japan) operated at 15kV. Cross-sectional samples were prepared by fracturing films in liquid nitrogen. Then, the samples were mounted on metal grids and coated with gold under vacuum. The cross-sectional morphologies were observed with 1000 and 2500x magnification.

4.3.11 Microbiological analysis

Antimicrobial activity testing of the films was carried out using the agar diffusion method (Chana-Thaworn *et al.*, 2011; Zivanovic *et al.*, 2005). A 48 hour old culture of *Saccaromyces cerevisiae* (*S. cerevisiae*) with 10^4 colony-forming units (CFU/mL) was spread on Hansen agar plates (0.1 mL/plate). While a 48 hour old culture of *Aspergillus oryzae* (*A. oryzae*) with 10^4 CFU/mL was spread on Crapek

nutrient plates (0.1 mL/plate). Film specimens were cut into 1.5 cm diameter discs and were then placed on the center of the plates. C/MC without carbendazim served as controls. The inhibitory effect of carbendazim concentration on *S. cerevisiae* and *A. oryzae* development was tested by counting the number of yeast and fungi developed on plates after incubation on agar nutrient at 25^{o}C for 3 days and observing the minimum radius of the fungi-inhibitory zone surrounding film discs. All experiments were done in triplicate.

The carbendazim content of each film specimen was calculated using the following equation (2):

$$X = \frac{CS}{A} \qquad (4.5)$$

Where: X is the carbendazim content in each film specimen (g)

C is the content of carbendazim in 50 mL C/MC film solution (g)

S is area of the film specimen with 1.5 cm diameter (1.76625 cm^2)

A is area of the glass plate with 14.5 cm diameter (165.0462 cm^2)

4.3.12 Statistical analysis

All the data were analyzed with one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's multiple range tests. Differences at $p = 0.05$ were considered as statistically significant (SPSS 16.0 software program).

Figure 4.2 The equipments were used to determine film properties included: (A,B): scanning electron microscope system; (C,D) oxygen permeability; (E): solubility and (F) prepare film samples for sorption isotherms determination.

4.4 Results and Discussion

4.4.1 Effect of various carbendazim content on film thickness, opacity and color

Films prepared from chitosan and methylcellulose incorporated with different carbendazim contents (0.8; 1.6; 3.2 and 4.8 g/100 g solid of C/MC are shown in Figure 4.3. The thickness of chitosan/methylcellulose film was 54.3 ± 3 µm. The thickness of the blended films depended mostly on the amount of carbendazim derivative added. Incorporating carbendazim into C/MC film increased the film thickness. When the content of carbendazim increased from 0.8 to 4.8 (g/100 g solid of C/MC), the thickness of films increased from 54.7 to 65.0 µm, respectively.

Table 4.2 represented color (L*, a*, b*, chroma; hue and opacity of films with varying carbendazim contents. Opacity is the opposite of transparency. A transparent material is defined as having a transmittance value above 90% or opacity less than 10%. The b* value represents the yellowish color of films. An increase in the b* value indicates that the color of the film is becoming more yellow. The L* parameter was used to describe the darkness to whiteness color with the ranged from 0 to 100. Chroma value shows intensity of color that change from neutral to dull and intensity when the chroma increases from 0 to 50 and 100, respectively. Hue parameter shows the actual color that altered from red-yellow to yellow and yellowish-green color when hue angle increased from 49° to 90° and 135°, respectively.

Chitosan/methylcellulose films without carbendazim and content 0.8 g carbendazim/100g solid of C/MC had percent opacity less than 10 which made a transparent film. The opacity of the films increased significantly with increase in content of carbendazim in the film-forming solution. The b* and chroma values increased slightly with increasing in carbendazim content means color of films changed to light white-yellowness and the intensity of color was increased (Table 4.2). Sangsuwan *et al*. (2008a) found that addition of vanillin into the C/MC resulted

in an opaque and yellow film. Zivanovic *et al.* (2005) indicated that chitosan film added the oregano essential oil became more opaque.

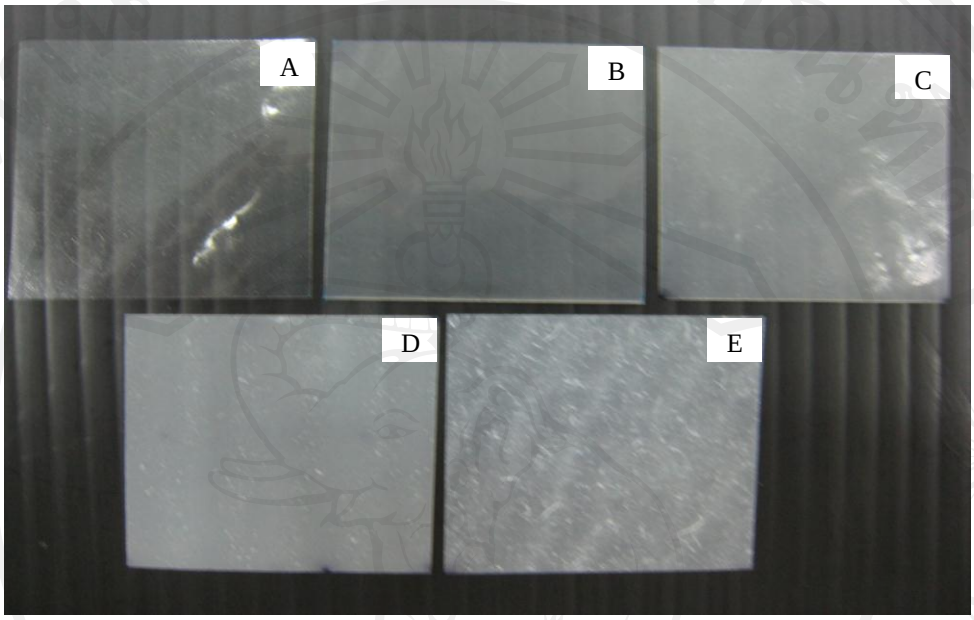


Figure 4.3 Films prepared from chitosan and methylcellulose incorporated with different carbendazim contents: (A) 0.0; (B) 0.8; (C) 1.6; (D) 3.2 and (E) 4.8 g/100g solid of C/MC.

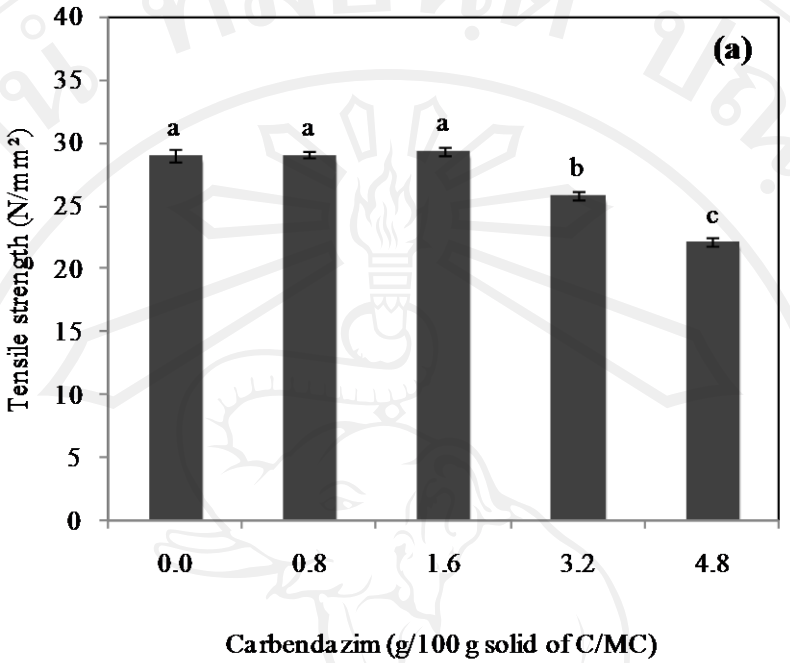
Table 4.2 Color and opacity of chitosan/methyl cellulose (C/MC) incorporated with different carbendazim contents

Parameters	Carbendazim contents (g/100g solid of C/MC)				
	0.0	0.8	1.6	3.2	4.8
L*	84.32 ± 0.04	83.72 ± 0.17	83.58 ± 0.04	83.16 ± 0.08	82.54 ± 0.08
a*	2.3 ± 0.00	2.08 ± 0.04	2.12 ± 0.04	2.4 ± 0.00	2.6 ± 0.00
b*	9.52 ± 0.10	11.9 ± 0.07	12.72 ± 0.12	13.16 ± 0.11	14.38 ± 0.14
Chroma	9.8 ± 0.07	12.1 ± 0.07	12.92 ± 0.14	13.38 ± 0.10	14.58 ± 0.12
Hue	76.36 ± 0.14	80.04 ± 0.14	80.58 ± 0.21	79.68 ± 0.12	79.78 ± 0.12
Opacity	8.61 ± 0.04	9.7 ± 0.02	10.56 ± 0.02	12.9 ± 0.05	17.92 ± 0.38

4.4.2 Effects of various carbendazim content on mechanical properties of C/MC films

The mechanical properties of the C/MC films were significantly ($p<0.05$) affected by high carbendazim concentration as an antimicrobial agent. The TS and %E of the C/MC incorporated with low carbendazim contents (0.8 and 1.6 g/100 g solid of C/MC) were not significant different from the control, however, the TS and %E of the films did decrease with increasing carbendazim concentrations of 3.2 to 4.8 g/100 g solid of C/MC (Fig. 4.4). For example, the TS and %E of the C/MC film without carbendazim was only 29.0±0.46 N/mm^2 and 61.5±0.77%, respectively and dropped to 22.1±0.46 N/mm^2 and 48.2±0.89%, respectively when carbendazim content increased to 4.8 g/100 g solid of C/MC. These results are similar to that of Sangsuwan *et al*. (2008a) who found that addition of vanillin as an antimicrobial agent into the C/MC film decreased the percent elongation, which is also related to the flexibility of such films. However, the C/MC films incorporated with 4% benzoate and sorbate showed increased TS and %E (Chen *et al.*, 1996).

Chitosan and methylcellulose films are hydrophilic because they contain hydroxyl groups which also makes them poor moisture barriers (Kamper and Fennema, 1984). The high tensile strength values were attributed to the numerous hydrogen bonds between the methylcellulose chains (Turhan *et al*., 2001). The decrease of mechanical properties of C/MC film with increasing carbendazim contents may be explained by the partial carbendazim insolubility at high content. When carbendazim was incorporated in to the film to create the incomplete solubilization, the carbendazim reduced hydrogen bonding between the MC chains which reduced the intermolecular attraction between the C/MC and the carbendazim leading to a decrease in the tensile strength and percent elongation.



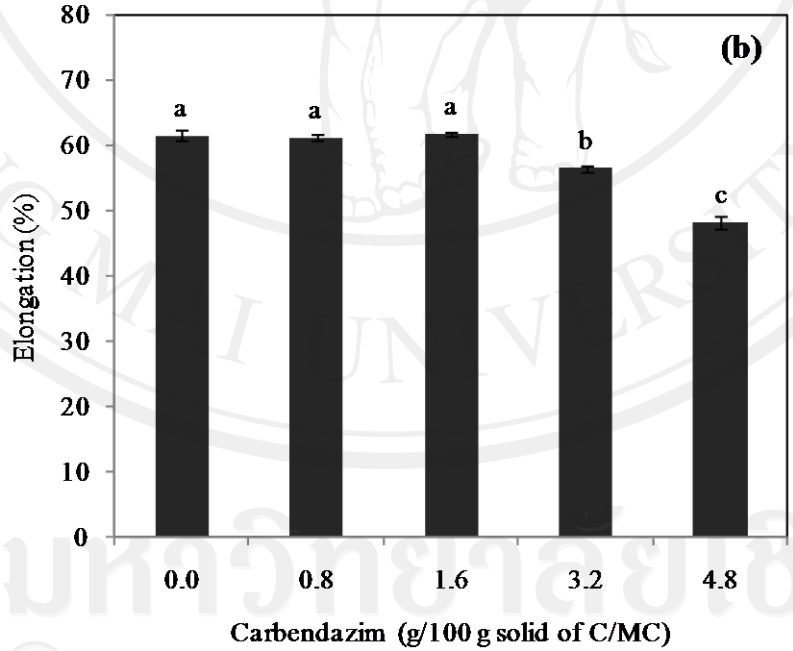


Figure 4.4 Effect of carbendazim content on (a) tensile strength; (b) percent elongation of chitosan/methylcellose films.

4.4.3 Effect of carbendazim concentrations on the water vapor permeability and oxygen permeability of C/MC films

The presence of carbendazim affected the water vapor permeability (WVP) of the C/MC films (Figure 4.5). The WVP was significantly increased at high carbendazim contents from 3.2 to 4.8 g/100 g solid of C/MC. The C/MC films, which contained the higher carbendazim, allowed significantly more water to pass through. For example, at 53% relative humidity condition, no significant difference in WVP was found between films prepared with 0-0.8g carbendazim, however, the WVP increased from $0.79x10^{-10}$ to $0.90x10^{-10}$ (g m/m^2 s Pa) when the carbendazim content increased from 3.2 to 4.8 g/100 g solid of C/MC (Figure 4.5a).

The WVP of chitosan films, methylcellulose films, chitosan/methylcellulose films and some antimicrobial films are reported in Table 1. Our results on the WVP of C/MC are similar to those reported by Caner *et al.* (1998), García *et al*. (2004), Sangsuwan *et al*. (2008b) and Wong *et al.* (1992). Garlic oil and vanillin, which are hydrophobic, were incorporated into chitosan films but did not significantly affect the WVP of these films (Pranoto *et al.*, 2005; Sangsuwan *et al.*, 2008a; Sangsuwan *et al.*, 2012). In contrast, carbendazim slightly increased the WVP which is necessary to preserve fresh fruits and vegetable, since they require respiration after being harvested. Compared to hydrophilic films based on soy protein and whey protein, the C/MC films with carbendazim added are better water vapor barriers by about ten times (Cho and Rhee, 2004; McHugh *et al.*, 1994).

The oxygen permeability (OP) of the C/MC films with and without carbendazim was shown (Figure 4.5b). At 23 ± 1^{o}C and 0% RH, the oxygen permeability of the C/MC films increased with increasing carbendazim contents. When carbendazim increased from 0.0 to 0.8 g/100 g solid of C/MC, the OP increased slightly from 2.27 to $2.36x10^{-12}$ cm^3cm/cm^2scmHg, respectively. However, the OP of these films increased significantly from 2.44 to $3.09x10^{-12}$ cm^3cm/cm^2scmHg for carbendazim contents ranging from 3.2 to 4.8 g/100 g solid of C/MC, respectively.

Table 4.3 WVP values of biodegradable films and antimicrobial films.

Film formulation	WVP (g m/m^2 s Pa)	References
Methylcellulose	0.87x10^{-10}	Donhowe and Fennema, (1993)
Methylcellulose 1%	0.75x10^{-10}	García *et al*. (2004)
Methylcellulose 3%	0.84-1.21x10^{-10}	Park *et al.* (1995)
Methylcellulose 1,5%	0.59x10^{-10}	Turhan *et al.* (2004)
Chitosan 1%	0.90x10^{-10}	García *et al.* (2004)
Chitosan 2%	0.36-0.48x10^{-10}	Wong *et al*. (1992)
Chitosan 3%	0.61-1.52x10^{-10}	Caner *et al.* (1998)
Chitosan/methylcellulose (1.0:0.5)	0.66x10^{-10}	García *et al.* (2004)
Chitosan/methylcellulose (1.5:0.5)	0.69x10^{-10}	Sangsuwan *et al*. (2008a)
Chitosan/methylcellulose (1.5:1.0)	0.77x10^{-10}	*Present study*
C/MC with vanillin	0.61-0.66x10^{-10}	Sangsuwan *et al.* (2008a)
C/MC with carbendazim	0.79-0.90x10^{-10}	*Present study*
Soy protein film	8.0-8.5x10^{-10}	Cho *et al.* (2004)
Whey protein plasticized with sorbitol	7.17x10^{-10}	Mc Hugh *et al.* (1994)

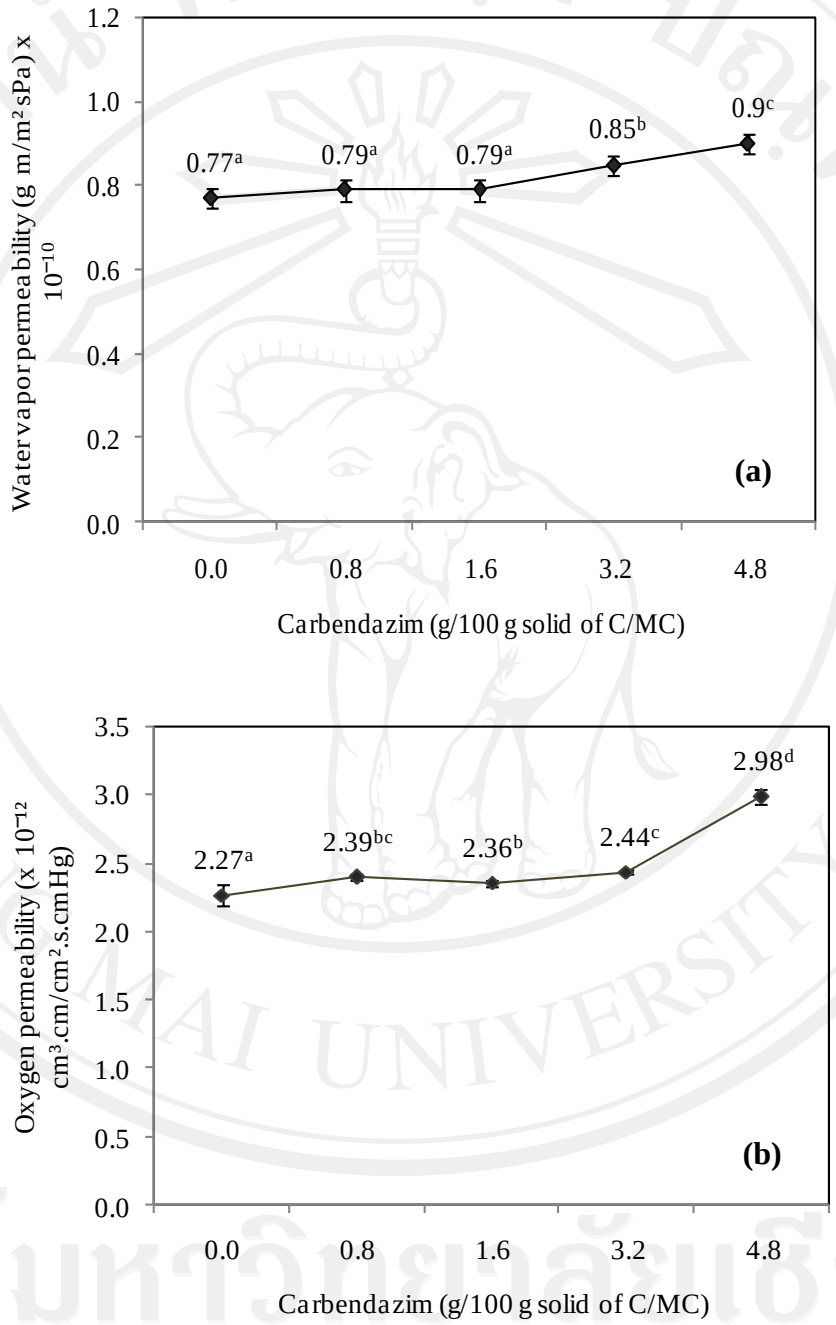


Figure 4.5 Effect of carbendazim content on (a) water vapor permeability; (b) oxygen permeability of chitosan/methylcellose films.

Increasing the permeability could be related to an increase in the diffusion coefficient in the films. Because permeability (P) is equal to the product of the diffusion coefficient (*D*), which represents the mobility of permeant molecules in the polymer, and the solubility coefficient (*S*), which represents the permeant concentration in the film in balance with the external pressure: $P = DS$ (Park and Chinnan, 1995). For high carbendazim content C/MC films, carbendazim reduces the hydrogen bonding between the MC chains which reduces the intermolecular attraction between the C/MC and carbendazim, due to structural changes in the polymer matrix. Therefore, the diffusion coefficient increased leading to an increase in the oxygen permeability of these films. Comparing our films to vanillin and some commercial plastics such as high-density polyethylene, low-density polyethylene, polypropylene and polystyrene, all films in our research proved to have a better oxygen barrier. The OP of vanillin film and polypropylene were 23.9-197.5x10^{-12} cm^3cm/cm^2scmHg and about 100x10^{-12} cm^3cm/cm^2scmHg, respectively (Sangsuwan *et al.*, 2008a; Smith, 1986).

WVP and OP are important properties of films when the films are used as a material packaging for food, fruits and vegetables. However high WVP and OP values much also might increase weight loss and spoilage by microorganism for food and fruits. With a suitable WVP and OP, the film may improve the shelf life of a product. Our results show that a high carbendazim concentration allows a significant amount of water and oxygen to pass through and thus the WVP and OP increased. Therefore, C/MC film blended with carbendazim from 0.8 to 1.6 g/100 g solid of C/MC have the desired properties.

4.4.4 Effects of carbendazim concentrations on morphology of C/MC

various carbendazim concentrations were investigated. The cross-section morphologies of films were observed with 1000x magnification (Figure 4.6) and 2500x magnification (Figure 4.7).

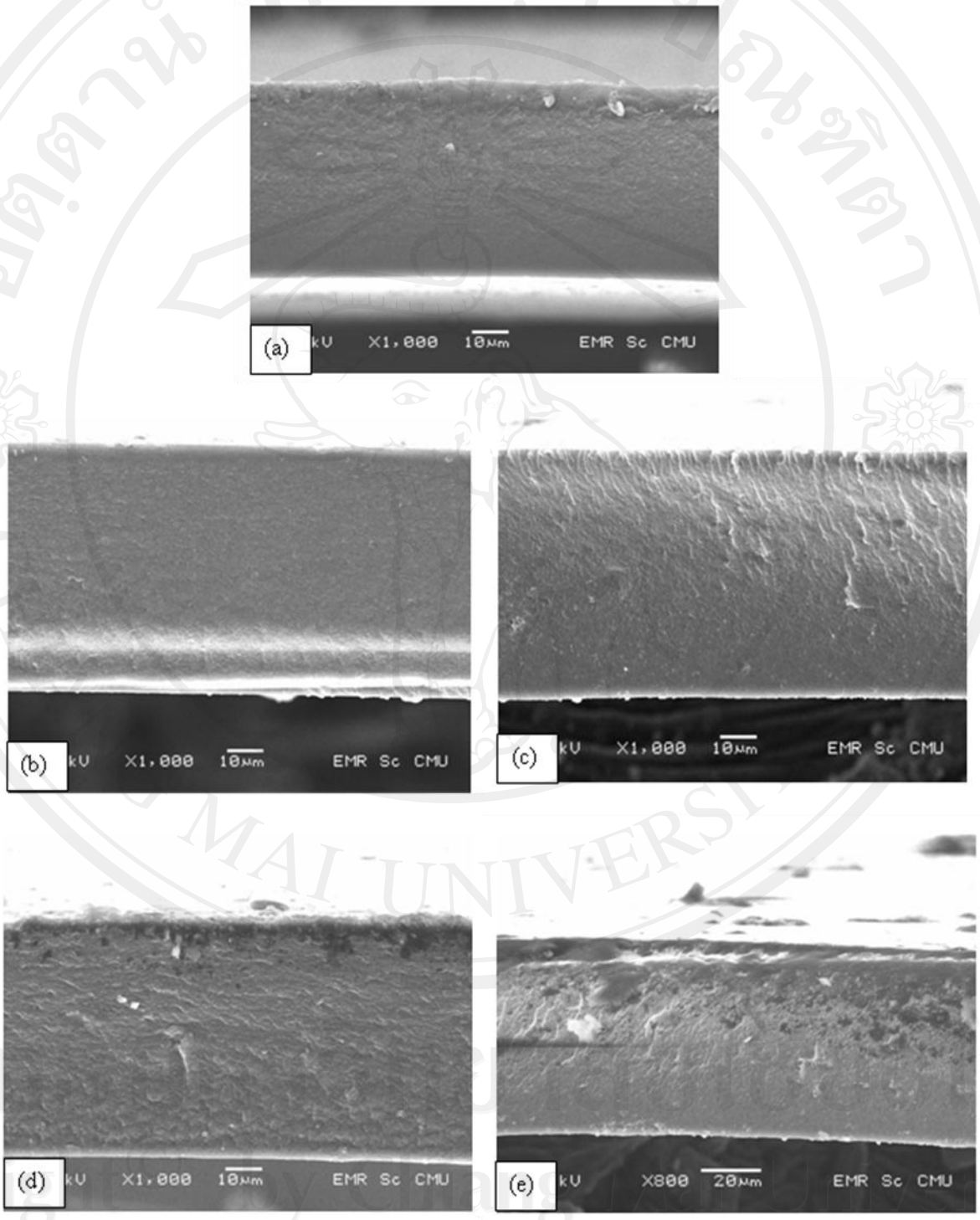


Figure 4.6 The cross-section morphologies of chitosan/methylcellose films incorporated with various carbendazim contents at 1000x: (a) 0.0; (b) 0.8; (c) 1.6; (d) 3.2 and (e) 4.8 g/100g solid of C/MC.

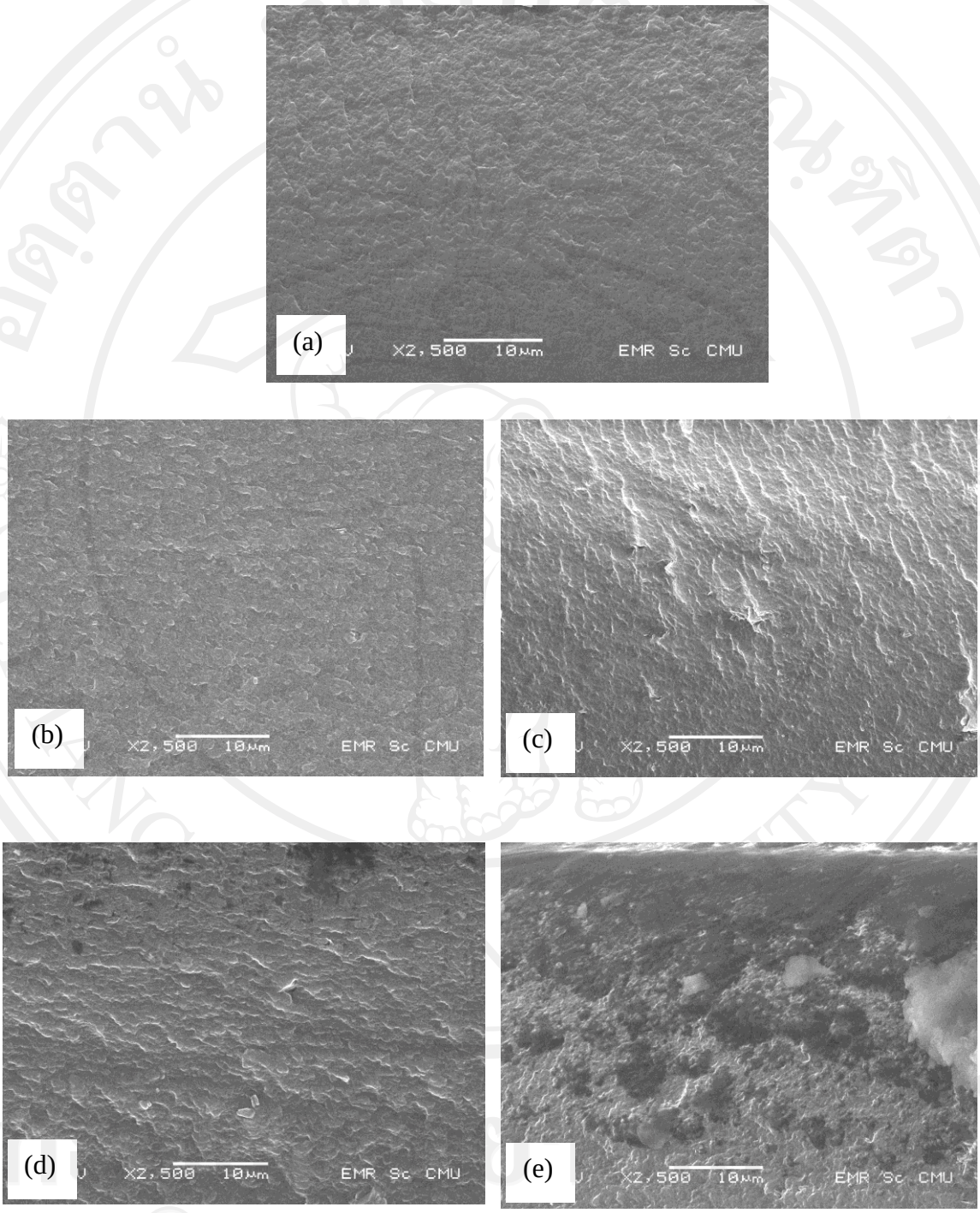


Figure 4.7 The cross-section morphologies of chitosan/methylcellose films incorporated with various carbendazim contents at 2500x: (a) 0.0; (b) 0.8; (c) 1.6; (d) 3.2 and (e) 4.8 g/100g solid of C/MC.

Our results suggest that with increasing carbendazim concentrations there was a significant change in the morphology of the cross-section of the films (Figure 4.6). The cross-section of C/MC films containing 0.0-0.8 g /100 g solid of C/MC carbendazim were smooth (Figure 4.6a,b) while the films became rougher and less homogenous at 3.3-4.8 (g) carbendazim concentrations (Figure 4.6 d,e). The cross-sectional morphology of C/MC films incorporated with 1.6 g carbendazim/100 g solid of C/MC was rougher but still homogenous (Figure 4.6c). Some phase-separations appeared in the cross-section morphology of film containing high carbendazim contents (Figure 4.6d,e). This result may be related to the limit of the carbendazim solubility in a C/MC solution. The presence of phase-separations of carbendazim in film created more holes between the C/MC and carbendazim molecules and the contact-surface between their molecules became non-smooth. This result indicates that the bonds between the C/MC and carbendazim molecules were reduced at high carbendazim contents and this property was related to the decrease of mechanical properties and the increase of water vapor permeability and oxygen permeability of films.

4.4.5 Effects of carbendazim concentrations on film solubility and sorption isotherms

The water permeability and water absorption properties of films are involved with the solubilization and diffusion of molecules through the film matrix. Therefore, the affect of carbendazim content on C/MC film solubility and moisture sorption isotherms modified the films solubility as is investigated. The C/MC films with carbendazim had lower solubility values than in the control film (Figure 4.8). The films solubility decreased from 64.3 to 56.9% as carbendazim content increased from 0 to 1.6 g/100 g of solid C/MC, respectively, and then increased slightly to 60.3% at 4.8 g/100 g of solid C/MC. The pure film incorporated with 1.6 g carbendazim achieved the lowest solubility value.

Moisture sorption isotherms for the C/MC films were determined in a controlled relative humidity system (0.11-0.86 a_w) using saturated salt solutions. For

all films stored in controlled RH desiccators and monitored over time. Moisture sorption increased rapidly in the initial stages and then lower amounts of water were absorbed with increasing time. When the moisture content of the films reached a plateau, this indicated the film's moisture had equilibrated with the relative humidity in each condition. At higher relative humidity's, an increased time was required to reach equilibrium. For films stored under 44.5% RH, the equilibrium was reached within 5 days while it took 7 days for films stored at 85% RH.

The change in the moisture sorption isotherms for C/MC films with and without carbendazim is shown in Figure 4.9. All of these films can be classified as having a type II sigmoidal isotherm. The equilibrium moisture content (EMC) of all of these films dramatically increased above a_w = 0.664. The presence of carbendazim affected the moisture sorption isotherms of the chitosan/methylcellulose films. C/MC films with carbendazim had lower EMC than in the control film (without carbendazim). The EMC of the C/MC films decreased when the level of carbendazim content increased from 0.8 to 1.6 g/100 g of solid C/MC. The C/MC film with 1.6 g/100 g of solid C/MC of carbendazim and had the lowest moisture content. However, the water absorption property of the C/MC films was not significantly different at higher carbendazim contents (3.2 to 4.8 g/100 g of solid C/MC). The sorption isotherm of the studied films were similar to those of cassava flour films (Kulchan *et al.*, 2010; Mali *et al.*, 2005), methylcellulose, ethylcellulose films (Velaquez de la Cruz *et al.*, 2001), and hydroxypropyl methylcellulose films (Villalobos *et al.*, 2006).

The change in the moisture sorption isotherms of C/MC films at different carbendazim contents may be related to changes in the morphology of the cross-section of the films (Figure 4.7). The cross-section of the C/MC films containing 0.0-0.8 g /100 g solid of C/MC carbendazim were smooth (Fig. 4.7a,b) while they became rougher and less homogenous at 3.3-4.8 (g) carbendazim contents (Figure 4.7d,e). Compared to C/MC films, the cross-section morphologies of C/MC films incorporated with 1.6 g carbendazim/100 g solid of C/MC were rougher but still homogenous (Figure 4.7c).

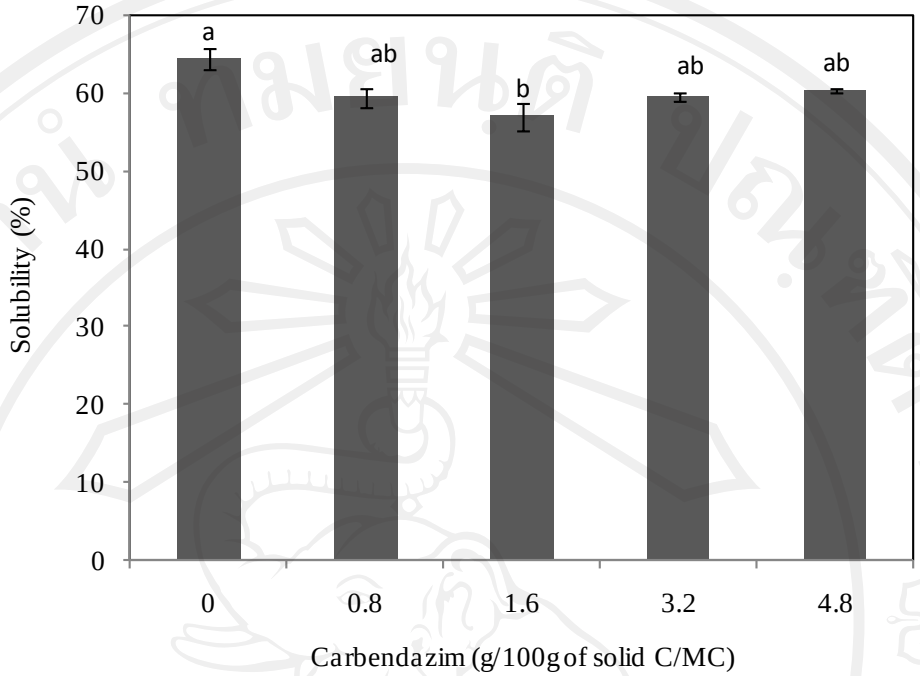


Figure 4.8 Solubility of chitosan/methylcellulose containing different levels of carbendazim at 25 ± 0.5°C.

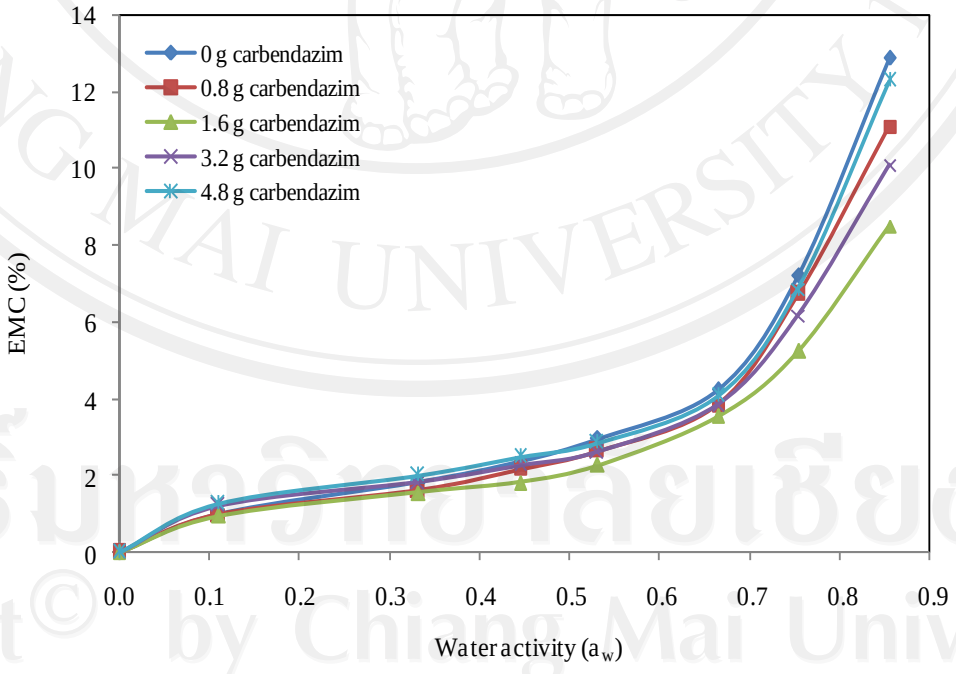


Figure 4.9 Moisture sorption isotherms of chitosan/methylcellulose films incorporated with carbendazim (g/100 g of solid C/MC) at 25 ± 0.5°C.

There was some partial carbendazim insolubility found in the C/MC film solutions. When carbendazim was blended into the films to create the incomplete solubilization, the distribution of carbendazim molecules can reduce the water absorption of chitosan molecules and methylcellulose chains, therefore the EMC decreased. Moreover, the results for the morphology of C/MC films with carbendazim showed that some small phase-separations appeared in the cross-sectional morphology of films containing high carbendazim contents (Figure 4.7c-e). The presence of small phase-separations of carbendazim in the films may be related to the change of moisture sorption isotherm of these films.

4.4.6 Antimicrobial activity of C/MC film incorporated with carbendazim

Carbendazim is a systemic fungicide that has commonly been used on several crops (pineapple, grapes, banana, cucumber, melons, lemon, oranges, strawberries, tomatoes, vegetables and cereals) (Anastassiades and Schwack, 1998; Medina *et al.*, 2007; Veneziano *et al.*, 2004; Zhang *et al.*, 2007). Carbendazim can inhibit the polymerization of free tubulin molecules by binding an arginin residue of the β-tubulin subunit which acts to disrupt cell division by linking to the nuclear spindle, thereby inhibiting fungal growth (Kling and Jakobsen, 1997).

The inhibitory effects of films containing varying amounts of carbendazim on yeast (*Saccaromyces cerevisiae*) and fungi (*Asperillus oryzae*) growth were observed on day 3 at 25^{o}C. *S. cerevisiae*, 2.7 x 10^4 CFU/mL, was spread on Hansen agar while *A. oryzae*, 2.1 x 10^4 CFU/mL was spread on Crapek nutrient. The increase in carbendazim content in C/MC film specimens was measured (Table 4.4). For carbendazim contents from 0.8 to 4.8 g/100 g solid of C/MC, the amounts of carbendazim per cm^2 and per film specimen (1.766 cm^2) increased to 0.03-0.18 mg and 0.05-0.32 mg, respectively. The presence of carbendazim and the increase of carbendazim contents in C/MC film specimens significantly affected the growth of *S. cerevisiae* (Fig. 4.10). The concentration of yeast decreased dramatically from $41.43\pm1.7x10^4$ to $15.71\pm0.8x10^4$ CFU/mL at carbendazim contents from 0.0 to 0.03 mg/cm^2 of film for means of 0.0-0.05 mg/film per specimen. The lowest amount of *S.*

cerevisiae was achieved $10.86 \pm 1.1 \times 10^4$ CFU/mL for a 0.06 mg carbendazim/cm^2 film. Our results show that there was no significant difference ($P<0.05$) in the anti-yeast activity of the C/MC films incorporated with carbendazim from 0.12 to 0.18 mg/cm^2.

Table 4.4 Carbendazim content in film-forming, film area (1 cm^2) and film specimen (1.766 cm^2).

Carbendazim (g/100g solid of C/MC)	Carbendazim (mg/cm^2 of film)	Carbendazim (mg/film specimen)
0.8	0.03	0.05
1.6	0.06	0.11
3.2	0.12	0.21
4.8	0.18	0.32

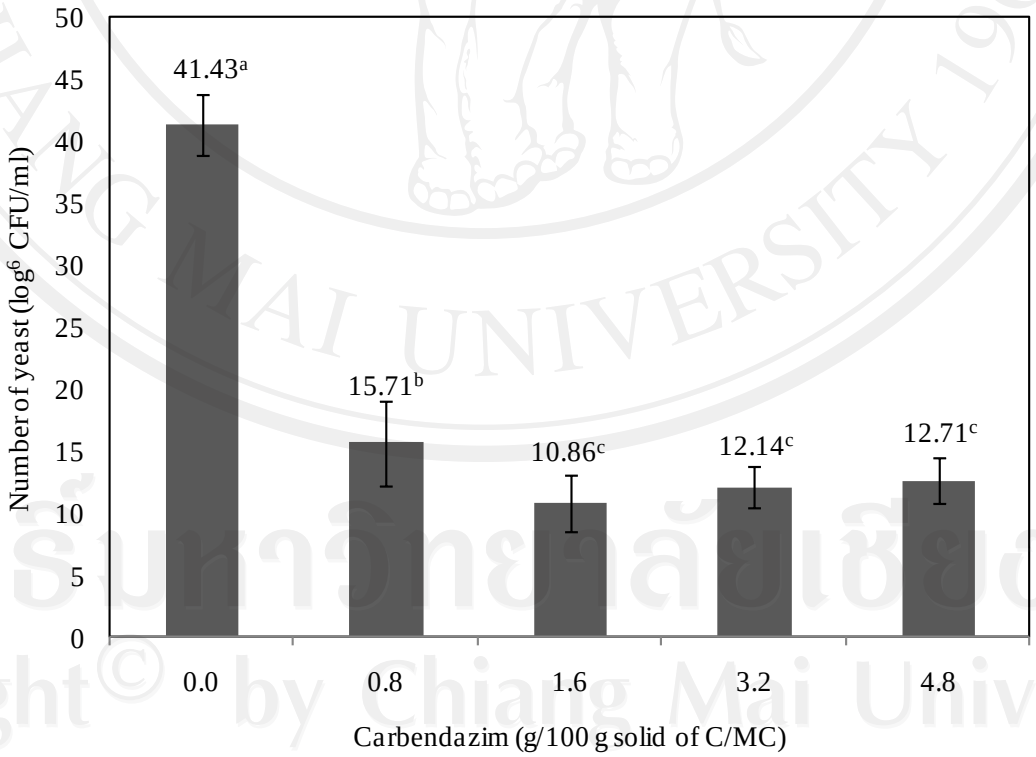


Figure 4.10 Inhibition effect of chitosan/methylcellulose film containing various amount of carbendazim against *Saccharomyces cerevisiae* after 3 days at 25°C.

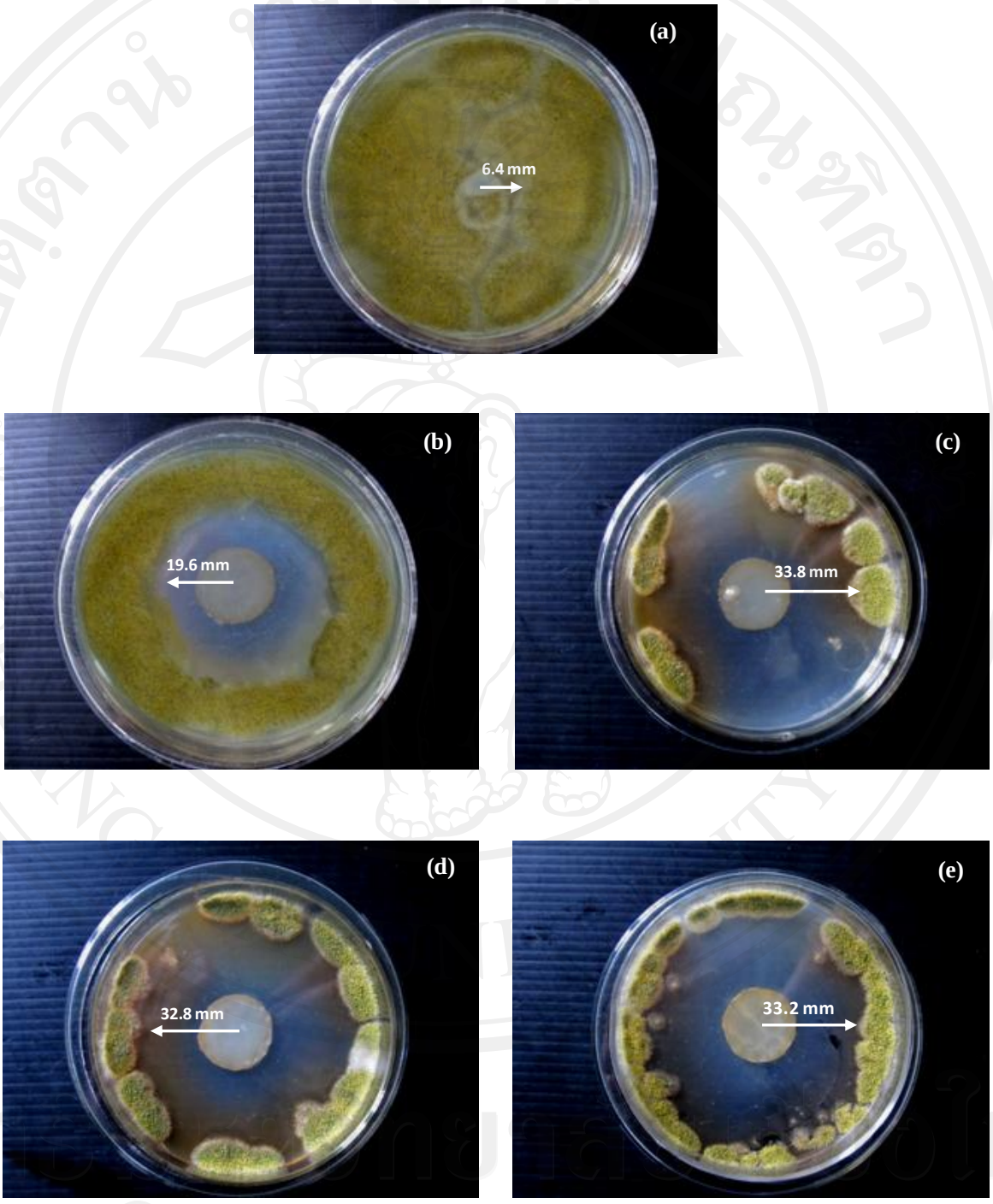


Figure 4.11 Inhibition effect of chitosan/methylcellulose films containing various amounts of carbendazim against *Aspergillus oryzae* after 3 days at 25°C: (a) 0.0; (b) 0.8; (c) 1.6; (d) 3.2 and (e) 4.8 g/100g solid of C/MC.

This result shows that increasing of the carbendazim amount more than 1.6 g/100 g solid of C/MC, means 0.06 mg/cm^2 of film or 0.11 mg/film specimen (Table 4.5) did not increase the inhibition effect of yeast development on these films. The influence of starch, whey protein, NaCl and oil on the antimicrobial effect of chitosan has also been investigated (Devlieghere *et al.*, 2004). Chitosan inhibited yeast growth at 0.01% (w/v). Starch, whey proteins and NaCl had a negative effect on the antimicrobial activity while oil conversely had no influence. C/MC films incorporating vanillin to provide protection against *S. cerevisiae* have also been reported (Sangsuwan *et al.*, 2008b). They reported that C/MC films containing low vanillin did not inhibit yeast while films containing medium and high vanillin partially inhibited the growth of yeast.

Table 4.5 Minimum inhibitory zone (mm) and number of fungi (CFU/ mL) developed on the C/MC film incorporated with carbendazim.

Carbendazim (g/100g solid of C/MC)	Minimum inhibitory zone (mm)	The number of mould ($\log^4$ CFU/ mL)
0.0	6.4 ± 0.34^a	31.2 ± 3.5^a
0.8	19.6 ± 0.33^b	19.9 ± 2.5^b
1.6	33.8 ± 0.38^c	5.7 ± 1.0^c
3.2	32.8 ± 0.44^c	6.9 ± 1.1^c
4.8	33.2 ± 0.41^c	6.3 ± 0.9^c

Alphabets in a column indicate significant difference at $p < 0.05$ by Duncan's multiple range test.

Similar to the inhibition effect on *S. cerevisiae*, the C/MC films incorporating carbendazim effectively inhibited the growth of *Aspergillus oryzae*. The minimum inhibitory radius around the film increased and the number of *A. oryzae* reduced significantly after 3 days incubated at 25^{o}C (Table 4.5). The minimum inhibition zone of the C/MC films without carbendazim was only 6.4 mm (Figure 4.11a) while this

zone of films contained carbendazim increased from 19.6 mm to 33.8 mm (Figure 4.11b,c,d,e). The C/MC film with carbendazim, 1.6 g/100 g solid of C/MC (which means 0.06 mg/cm^2 of film or 0.11 mg/film specimen) was the best antimicrobial film, which showed the biggest inhibitory zone (Figure 4.11c) and the lowest number of *A. oryzae* (Table 4.5). Our results also showed that an increase in carbendazim more than 1.6 g/100 g solid of C/MC did not significantly change the antimicrobial activity of the film.

4.5 Conclusions

The results in this study show that carbendazim could be used as an antimicrobial agent for chitosan/methylcellulose film properties. The TS and E% were found to decrease while the WVP and OP increased for increasing carbendazim content. Higher carbendazim contents, from 3.2 to 4.8 g/100 g solid of C/MC, strongly changed the C/MC film properties. The change of the mechanical properties and the permeability of water and oxygen also had a strong relationship with the film morphology. Moreover, C/MC films incorporated with carbendazim were shown to be a good antimicrobial agent which could control the growth of yeast (*S. cerevisiae*) and fungi (*A. oryzae*) at 25^{o}C. The carbendazim films of 0.03-0.18 mg/cm^2, incorporated with carbendazim, at 1.6 g/100 g solid of C/MC, were found to be the best film for use as an antimicrobial material for fresh fruit, fresh vegetable and the food-packaging industry.